VOLUME 51 [J. CELL. AND COMP. PHYSIOL.] NUMBER 3

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THIRTY-SIXTH STREET AT SPRUCE, PHILADELPHIA 4, PA.

Second class postage paid at Philadelphia, Pa.

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A QUANTITATIVE INTERPRETATION OF ESERINE-ACETYLCHOLINE INTERACTION IN THE FROG RECTUS ABDOMINIS[1]

DONALD J. JENDEN[2]
WITH THE TECHNICAL ASSISTANCE OF CHARLES E. KNIGHT
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TWO FIGURES

It is well established that eserine in small concentrations potentiates the effect of acetylcholine (ACh) on the rectus abdominis muscle of the frog by reversibly inhibiting cholinesterase (ChE). The purpose of the present work was to examine this potentiation quantitatively and to analyze the experimental results in terms of the component rate processes of diffusion and enzymatic destruction of ACh. From such an analysis it has been possible to estimate the dissociation constant between eserine and ChE in an intact muscle.

THEORY

(*a*) *Concentration-response relation in the muscle.*

The sigmoid curve relating concentration of ACh to the degree of shortening of the muscle after equilibration has been interpreted as Gaussian (Smith et al., '52) and as logistic (Clark and Raventos, '37). Which interpretation is used is probably of little significance, since the difference is small (Finney, '52) and the experimental errors relatively large. However, the normal distribution is more likely to govern a quantized system with statistically distributed thresholds or sensitivities, while the frog rectus shows a response in each fiber which is a graded function of concentration (Kirsch-

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[1] The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY, VOL. 51, NO. 3
JUNE 1958

ner and Stone, '50–51). In the present work a logistic relationship between ΔE, the response of any part of the muscle, and log u_r, where u_r is the concentration of ACh at the endplate, has been assumed. Expressing the relation in its integral form,

$$C \cdot u_r^n = \frac{\Delta E}{\Delta E_{max} - \Delta E} \qquad (1)$$

where c and n are constants and ΔE_{max} is the maximum response to ACh of which the muscle segment is capable.

(b) *ACh concentration at the endplate if ChE forms a flat layer over it.*

In the absence of accurate information concerning the distribution of ChE in the neighborhood of the endplate, the simplest arrangement will be assumed, namely, that the endplate is covered with a flat homogeneous ChE-containing layer of d cm thickness. It will further be assumed that ACh does not diffuse past the endplate, i.e., into the muscle fiber.

All the experiments in this work were carried out at ACh concentrations less than 10^{-5} M, and since this is very small compared with the Michaelis constant of ChE (2.6×10^{-4}: Wilson and Bergmann, '50), it will be assumed that first order kinetics apply, both for the uninhibited and the inhibited enzyme. Diffusion through the ChE-containing layer can now be described by the following equation for the steady state:

$$k \frac{d^2u}{dx^2} = mu \qquad \begin{matrix} u(o) = u_E \\ \frac{du}{dx}(d) = 0 \end{matrix} \qquad (2)$$

where k is the diffusion coefficient of ACh through the ChE-containing layer, x is distance normal to the surfaces of this layer, $x = 0$ at the outer surface and $x = d$ and the endplate, u is the concentration of ACh and $u = u_E$ at the surface of the layer, and m is the first order rate constant of hydrolysis. The solution is:

$$u = \frac{u_E \cosh\left\{(d - x)\sqrt{\frac{m}{k}}\right\}}{\cosh\left\{d\sqrt{\frac{m}{k}}\right\}} \qquad (3)$$

At the endplate, $x = d$, so that

$$u_r = \frac{u_E}{\cosh\left\{ d\sqrt{\frac{m}{k}} \right\}} \tag{3a}$$

(*c*) *The response of the muscle in terms of cholinesterase activity and outside concentration of acetylcholine.*

If equation 3a is used to substitute for u_r in equation 1, then on rearranging we have

$$\frac{\Delta E}{\Delta E_{max}} = \frac{C \cdot u_E^n}{C \cdot u_E^n + \cosh^n\left\{ d\sqrt{\frac{m}{k}} \right\}} \tag{4}$$

If the overall rate of ACh hydrolysis is insufficient to affect materially the general extracellular concentration, u_E can be regarded as constant throughout the muscle; furthermore, if all the endplates are similar the quantity $d\sqrt{m/k}$ will also be independent of position, so that $\Delta E/\Delta E_{max}$ will be the same everywhere in the muscle and therefore equal to E/E_{max}, where E is the response of the entire muscle. We can therefore write

$$\frac{E}{E - E_{max}} = \frac{C \cdot u_{EE}^n}{\cosh^n\left\{ d\sqrt{\frac{m}{k}} \right\}} \tag{4a}$$

where u_{EE} is the ACh concentration outside the muscle.

If the quantity $\log_{10} \left(\frac{E}{E_{max} - E}\right)$ is plotted against $\log_{10} u_E$, a straight line should result with a slope of n, intersecting the x axis at $\log_{10} u_E = \log_{10} \cosh\ (d\sqrt{m/k}) - \frac{1}{n} \log_{10} C$. If the subscript zero indicates the condition when all the ChE has been inactivated, i.e., $m = 0$, and U is the concentration of ACh corresponding to the x axis intercept,

$$\frac{U}{U_0} = \cosh\left\{ d\sqrt{\frac{m}{k}} \right\} \tag{5}$$

Similarly, if partial inactivation of ChE is denoted by the subscript i,

$$\frac{U_i}{U_0} = \cosh\left\{ d\sqrt{\frac{m_i}{k}} \right\} \tag{5a}$$

When the ACh concentration-response data from experiments in which varying amounts of anticholinesterase are used are plotted in this way, a family of parallel lines should therefore be obtained, from the intercepts of which the relative values of m may be obtained, since both d and k may be regarded as constant in a given muscle. It is necessary, however, to know the ACh sensitivity after complete ChE inactivation in order to determine U_0 directly.

(*d*) *Estimation of K_I of an anticholinesterase.*

Equations 5 and 5a may be combined and the constants d and k eliminated to give

$$\frac{m}{m_i} = \left\{ \frac{\operatorname{ar\,cosh} \dfrac{U}{U_o}}{\operatorname{ar\,cosh} \dfrac{U_i}{U_o}} \right\}^2 \tag{6}$$

and since m and m_i represent first order rate constants of the enzyme, their ratio also represents the ratio of velocities at which hydrolysis would proceed under identical conditions. This ratio is related to the concentration of a reversible inhibitor by the following relationship (Wilson, '49) where [I] is the inhibitor concentration:

$$\frac{V}{V_i} = 1 + \frac{K_s}{K_I}\left\{\frac{[I]}{K_s + u}\right\} \tag{7}$$

Nachmansohn and Wilson ('51) have shown that this equation fits the inhibition of isolated electric eel cholinesterase by eserine. In the present experiments $K_s \geqq u$, and the equation reduces to

$$\frac{V}{V_i} = 1 + \frac{[I]}{K_I} \tag{7a}$$

From this, the K_I in the present experiments may be estimated.

(*e*) *Response of the muscle if the rate of ACh hydrolysis is fast enough to lower the general extracellular concentration.*

The frog rectus abdominis may be regarded as a flat sheet which is thin compared with its surfaces; diffusion will therefore be assumed to occur normal to the surfaces only, the ends and edges being insignificant. The ChE activity will be assumed to be constant throughout the muscle. The ACh concentration at steady state will be described by the equation

$$k_1 \frac{d^2u_E}{dx^2} = m_1u_E \qquad u_E(0) = u_E(2b) = u_{EE} \tag{8}$$

where k_1 is the effective diffusion constant of ACh through the muscle, m_1 is the first order rate constant of hydrolysis, averaged throughout the muscle substance, x is distance normal to the surfaces and $x = 0$ and $x = 2b$ at the surfaces. u_{EE} is the concentration at the surfaces and u_E is the concentration at x. The solution is

$$u_E = u_{EE} \frac{\cosh\left\{(b - x)\sqrt{\frac{m_1}{K_1}}\right\}}{\cosh\left\{b\sqrt{\frac{m_1}{K_1}}\right\}} \tag{8a}$$

If this expression for u_E is inserted into equation 4, and the resulting expression for ΔE is integrated between the limits 2b and zero, the approximate response of the entire muscle is obtained:

$$\frac{E}{E_{max}} = C' \int_0^{2b} \frac{U_{EE}^{n} \cosh^n\left\{(b - x)\sqrt{\frac{m_1}{K_1}}\right\}dx}{\cosh^n\left\{b\sqrt{\frac{m_1}{K_1}}\right\} + C'u_{EE}^{n} \cosh^n\left\{(b - x)\sqrt{\frac{m_1}{K_1}}\right\}} \tag{9}$$

where $c' = \dfrac{C}{\cosh(d\sqrt{m/k})}$

This expression is only integrable for certain specific values of n; however, if $m = 0$, inspection shows that for reasonable values of n (1.5), c (3.7×10^{10}) and $b\sqrt{m_1/k_1}$ (0–9), the concentration-effect plot described produces a family of lines with increasing curvature and decreasing slope as $b\sqrt{m_1/k_1}$ increases (fig. 1), and if $d\sqrt{m/k} > 0$, these lines will all be shifted to the right by the quantity $\log_{10} \cosh(d\sqrt{m/k})$. It is clear, therefore, that deviation from parallelism in the ACh dose-response curves obtained at different inhibitor

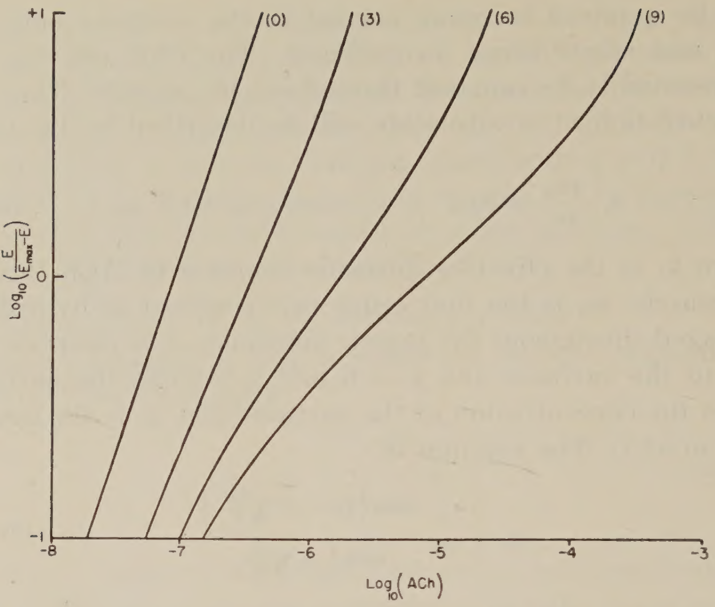

Fig. 1 Theoretical ACh dose response curves calculated by numerical integration of equation 9, with c′ 3.7×10^{10}, n 1.5. The figures in brackets refer to the values of b $\sqrt{m_1/k_1}$ from which each line was calculated. This type of dose response curve should be obtained if ACh hydrolysis is fast enough to lower the general extracellular concentration appreciably.

concentrations can be explained as due to an effect on the general extracellular concentration of ACh.

METHODS

Frogs (*Rana pipiens*) of both sexes, weighing 30–70 gm, were used in experiments performed in the months of April, May and June. The frogs were pithed, one or both rectus abdominis muscles were removed and suspended in a 20 ml bath, and their concentrations were recorded using an isotonic lever of sixfold magnification. The experiments were conducted in an air-conditioned room at 20°C without other means of temperature control. The solution used was that described by Boyle and Conway ('41) as being equivalent to frog plasma in electrolyte composition:

NaCl	4.240 gm/liter
$NaHCO_3$	2.100 gm/liter
$NaHPO_4$	.356 gm/liter
KH_2PO_4	.068 gm/liter
KCl	.148 gm/liter
$MgSO_4 \cdot 7H_2O$	.296 gm/liter
Na_2SO_4 (Anhydrous)	.092 gm/liter
Ca gluconate	.400 gm/liter
Glucose	4.684 gm/liter

This solution was made up daily from the stock solutions described by Boyle and Conway, and the glucose was added immediately before use. Oxygen with 3.0 ± 0.1% (limits) carbon dioxide was used for aeration, and the solution was equilibrated with this mixture for at least 30 min. before use.

RESULTS

In the absence of any anticholinesterase, the contracture produced by ACh generally reached within 2% of its final level in less than 4 minutes. This time was somewhat longer when the ChE was completely inhibited by DFP. Accordingly, 5 minutes were allowed for equilibration in the normal preparation and 6 minutes or longer if necessary after DFP. Between successive tests, the preparation was washed three times: 7, 5 and 1 minute before the next ACh dose was introduced. This schedule was found to be sufficient to enable the muscle to regain its original length and responsiveness, provided that the previous dose was less than 80% of maximum. The ACh concentrations used were chosen to give responses between 10% and 80% as far as possible because of the large measuring errors at extreme responses, particularly near maximal. The maximal response was determined at the end of each series of contractures by using an ACh concentration of 12.5×10^{-6} gm/ml. Under these conditions the ACh concentration-response curve was closely reproducible over a period of 8 hours (fig. 2). The sensitivity varied little between different preparations, and was generally such that a 50% of maximum contracture was produced by about 10^{-6} gm/ml ACh in the absence of anticholinesterase.

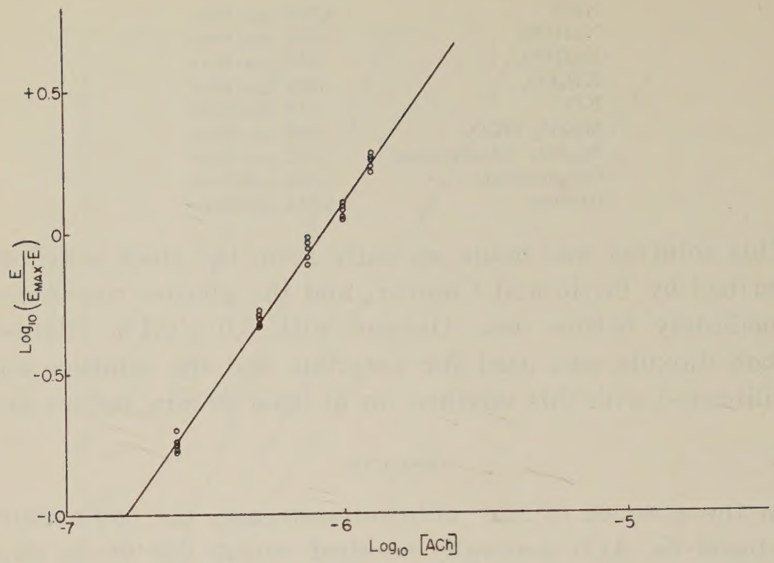

Fig. 2 Dose-response data repeated five times on the same preparation without anticholinesterase to show reproducibility. The line was obtained by least squares.

It was shown above that in order to arrive at an estimate of the relative ChE activity before and after partial inhibition, it is necessary to determine the ACh dose-response curve after complete inhibition as well. An attempt was made to achieve complete ChE inhibition by using a large excess of eserine, but it was found that 10^{-4} M eserine or more produced not potentiation of ACh but antagonism. This was interpreted to mean that at these concentrations the neuromuscular blocking action of eserine which has been described (Kirschner and Stone, '50–51; Quilliam and Strong, '49) was paramount, and in order to obtain complete ChE inhibition from this action it was decided to use DFP. A concentration of 1.09×10^{-4} M was used, and the preparation was equilibrated with it for at least one hour with two changes of bath fluid. Since longer exposure times produced no further potentiation it was concluded that inactivation was complete. No chemical confirmation was obtained. The DFP was maintained in the same concentration in the bath while the ensuing series of contractures were recorded, since there was strong evidence of a considerable degree of recovery of ChE after removal of the DFP under these conditions. Similar evidence of recovery after DFP was obtained from the rectus abdominis of *Bufo bufo* by Bacq ('49).

In the usual experiment, three ACh concentration-response curves were obtained, the first as a control, the second in the presence of eserine sulphate and the third after exposure to DFP. At least 5 ACh concentrations were used for each curve, including the large dose necessary to produce the maximal contracture. A period of 80 min. was allowed between the control and eserine series for equilibration, with at least two changes of bath fluid in order to ensure that the eserine concentration in the bath did not change as a result of binding by the preparations. In addition several rinses were used to remove all ACh before the eserine was added. A similar procedure was used between the second and third series, the eserine being removed before the DFP was added.

The results of each experiment were plotted as described in the theoretical section above, and an analysis of variance was carried out to determine whether there were significant deviations from parallelism. No attempt was made to assign weights to the points in this analysis, since only the central part of the response range was used; the tabulated weighting coefficients for logit analysis are not valid in this case because the maximum effect is not known exactly but is a measurement subject to error. Table 1 and figure 3 show the

TABLE 1

Analysis of variance for the experiment shown in figure 3

SOURCE	SUM OF SQUARES	D. F.	MEAN SQUARE	F	P
Regression	4.088601	1	4.088601	2017.9	.001
Deviation from parallelism	.003307	2	.001653	—	.2
Between drugs	.858997	2	.429499	212.0	.001
Residual (error)	.020261	10	.002026	—	—
Total	4.971166	15			

results of one of these experiments. The lines are parallel lines estimated by least squares, and the x axis intercepts can be used to estimate the K_I of eserine from equations 6 and 7a. Table 2 summarizes the results of twenty experiments, of which 6 showed significant deviation from parallelism. In all these the slope after treatment with DFP was greater than that in the untreated preparation, suggesting that in these cases the assumption that the ACh concentration is constant throughout the extracellular space is incorrect (cf. fig. 1). No attempt was therefore made to deduce further information from these experiments. The mean value of K_I was 11.4 ± 0.4 (S.E.) $\times 10^{-7}$. It will be noted that when the dose response curve after eserine approximates to the con-

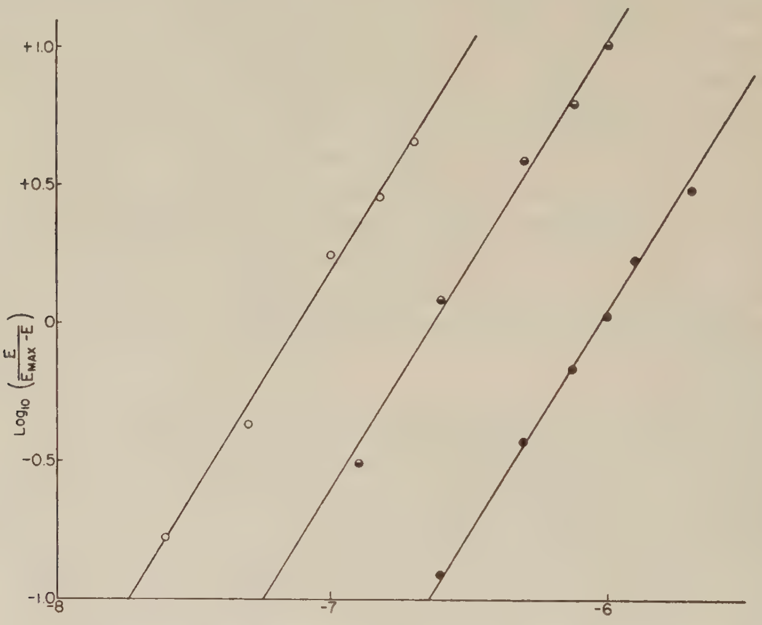

Fig. 3 The results of one experiment to determine the K_I of eserine and ChE in the rectus abdominis. Closed circles denote control data; open circles are data obtained after DFP; half-closed circles after eserine, 23.1×10^{-7} N. The lines are parallel lines fitted by least squares, and the analysis of variance is given in table 1. U/U_0 is 12.3 and U/U_1 is 3.99.

trol or to that after DFP, the accuracy of the estimate of K_I falls off, and greater variation is therefore to be expected at very high and very low eserine concentrations.

In 6 additional experiments the effect of two different concentrations of eserine were determined instead of using a single eserine concentration followed by DFP. The K_I was then estimated by finding by trial and error the value of U_0 which gives identical values for K_I for the two eserine concentrations. The results are summarized in table 3, and it can be seen that good agreement was obtained with the direct determinations, the mean value being 10.9 ± 0.6 (S.E.) $\times 10^{-7}$.

TABLE 2

Results of 20 experiments in which the effect of eserine was compared with the effect of DFP in maximal dose on sensitivity to ACh

U/U_0 is potentiation factor with DFP: U/U_I is potentiation factor with eserine. For further explanation see text.

ESERINE CONCENTRATION $\times 10^{-7}$ M	$\frac{U}{U_0}$	$\frac{U}{U_I}$	$\frac{U_I}{U_0}$	$\frac{m}{m_I}$	$K_I \times 10^{-7}$	P (Deviation from parallelism)
7.7	12.7	2.08	6.12	1.68	11.3	$> .20$
7.7	44.7	3.98	11.22	—	—	$< .001$
7.7	25.8	2.65	9.73	1.77	10.0	$> .05$
15.4	15.6	2.81	5.56	2.05	14.7	$> .10$
15.4	12.1	3.89	3.11	—	—	$< .05$
15.4	13.1	3.74	3.50	2.88	8.2	$> .10$
15.4	29.6	4.63	6.40	—	—	$< .01$
15.4	36.4	4.80	7.59	2.49	10.3	$> .20$
23.1	12.3	3.99	3.08	3.20	10.5	$> .20$
23.1	37.8	6.46	5.85	3.11	10.9	$> .10$
23.1	18.4	5.88	3.13	—	—	$< .05$
23.1	40.3	6.76	5.96	3.16	10.7	$> .05$
30.8	11.8	4.33	2.72	3.65	11.6	$> .20$
30.8	36.4	8.14	4.47	3.87	10.7	$> .10$
30.8	26.0	5.91	4.40	3.34	13.2	$> .05$
46.2	26.9	4.07	4.15	—	—	$< .05$
46.2	14.1	5.95	2.37	4.94	11.7	$> .20$
61.7	12.0	6.06	1.98	5.87	12.7	$> .20$
61.7	15.7	7.15	8.20	5.84	12.7	$> .05$
61.7	28.0	8.51	3.29	—	—	$< .02$

TABLE 3

Results of 6 experiments in which two eserine concentrations were used in each preparation

U/U_o and K_I were estimated trial and error from equations 6 and 7a.

ESERINE CONCENTRATION $\times 10^{-7}$ M	$\frac{U}{U_I}$	$\frac{U}{U_o}$ (Estimated)	$K_I \times 10^{-7}$
15.4	4.44	26.1	9.7
30.8	7.38		
15.4	3.45	18.6	11.6
61.7	8.35		
7.7	2.33	12.2	8.9
23.1	4.38		
30.8	5.55	17.1	10.8
61.7	8.22		
15.4	3.26	14.7	11.1
30.8	3.01		
15.4	3.64	24.2	13.4
46.2	7.50		

DISCUSSION

There is a good deal of evidence that the ChE in voluntary muscles is largely confined to the vicinity of the endplates (Marnay and Nachmansohn, '38; Augustinssohn, '48), although the details of its distribution around each endplate are not accurately known. In this paper the simplest model of its local distribution is used, and the endplates are regarded as having equal sensitivities and dimensions throughout the muscle. Although this is not likely, no great error should be introduced unless there is a correlation between endplate sensitivity and the thickness of the ChE layer. While the ChE distribution around the endplate may not be the flat layer assumed, the equation used would apply as an approximation for any distribution in which the thickness of the layer is small compared with the area it covers, and in which ACh can only gain access to the endplate by diffusing through the layer. The assumption that the concentration gradient of ACh at the endplate is zero when a steady state is achieved appears to be justified by the evidence that ACh cannot penetrate into the muscle fiber (Riker, '53).

It was shown in the theoretical section that if the ACh concentration throughout the extracellular space is approximately constant except in the immediate vicinity of the endplates, the ACh dose-response curves using a logistic plot should be parallel. If on the other hand the ChE were less strictly localized, the overall rate of hydrolysis might be fast enough to produce a concentration gradient of some magnitude in the extracellular space. This would alter the shape of the dose-response curves, which would become flatter in the 20–80% range as the ChE activity increased (fig. 1).

The results of the present work enable a distinction to be made between these two possible mechanisms of action of eserine. Inasmuch as the dose-response curves were straight and parallel in the majority of experiments, it appears that in these the ACh potentiation resulted from inactivation of the ChE layer covering the endplate. Although deviation from parallelism in the remaining 6 experiments indicates a significant effect on the ACh concentration in the general extracellular space in these preparations, it does not seem likely that this is the primary factor responsible for potentiation. A point of some importance in experimental design is that this factor may be minimized by using muscles which are as thin as possible.

It has been suggested (Smith et al., '52) that the negative logarithm of the concentration of potentiator required to multiply the sensitivity by a factor of x (pS_x) be used as a measure of potentiating activity analogous to Schild's ('47) symbol pA_x for expressing the activity of an antagonist. While this method of describing activity has many advantages, notably its general applicability, it is clear from table 2 that it would in this case be much more variable than the estimate of K_I. Moreover the latter is of more basic significance and can therefore be compared more usefully with estimates of anticholinesterase activity obtained in other ways. However, it is evident that a valid estimate of the K_I from potentiation studies can only be obtained when all potentiation results directly from enzyme inhibition.

The estimate of the K_I of eserine arrived at in the present work differs by a factor of almost 20 from the figure of 6.1×10^{-8} obtained from electric eel ChE (Nachmansohn and Wilson, '51). It is possible that this difference can be explained in part on the basis of species difference in the enzyme; no figures for the K_I of eserine and frog muscle ChE are available. On the other hand, ChE at the endplate is, like that obtained from the electric eel, true cholinesterase or acetylcholinesterase and the properties of the enzyme are to a large extent independent of species (Nachmansohn and Wilson, '51). It is perhaps more likely that the ChE in the living muscle is bound in a manner which changes the properties of specificity and affinity for receptors. The properties of the inhibitor and even of the substrate may also differ markedly from those shown in free solution. It may also be significant that the substrate concentration in the present experiments is substantially lower than can be used with a chemical method of determination. Finally, the method used here for the analysis of the experimental data is based on many assumptions and approximations which could lead to errors in the final results and may explain part or all of the difference from results on the isolated enzyme.

SUMMARY

The potentiation of acetylcholine by eserine on the frog rectus abdominis has been examined quantitatively in the light of the diffusion kinetics of acetylcholine in the muscle. The data are consistent with the localization of cholinesterase to the immediate vicinity of the endplate, and suggest that potentiation results mainly from inactivation of this barrier. In some experiments, there was evidence the average concentration of ACh in the extracellular space was lowered by hydrolysis by ChE, but generally this was not detectable.

The dissociation constant between ChE and eserine was estimated at $11.2 \times 10^{-7} \pm 0.7$ $(p = 0.05)$. This is approximately 20 times greater than published estimates on isolated acetylcholinesterase, and the possible reasons for this discrepancy are discussed.

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THE EFFECT OF X-IRRADIATION UPON POTASSIUM RETENTIVITY OF YEAST[1]

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FOUR FIGURES

Previous studies in this laboratory (Bair and Stannard, '55a, '55b; Bair, Stannard and Bruce, '56) have indicated a relationship between potassium transfer in yeast and changes in metabolic activity induced by irradiation. Recently we (Bair et al., '56) have shown by direct measurements that there is a marked increase in potassium leakage from yeast following x-irradiation but few details could be included in this preliminary report. Also, there has been essentially no indication as to the specificity or nature of the effect. This paper presents evidence for a dependence of the measured effects of x-irradiation on potassium retentivity of yeast upon the nature of the substrate utilized and the presence of oxygen in the medium, along with the experiments showing the relative specificity of the radiation effect on ion transport.

MATERIALS AND METHODS

Ion exchange. Measurements of ion exchange were made employing a column elution technique, as devised by Rothstein and Bruce ('58a) which allows maintenance of a constant extracellular environment during the experiment. From 500 to 530 mg of yeast was placed, after washing in distilled water and starvation for three hours with aeration, in a Buchner-type funnel of 30 mm diameter which contained a fritted disc of fine porosity. The yeast was sucked dry to form a cake, a sheet of filter paper and a wad of glass wool placed above the cake, and test solutions passed through the column at about 6 psi pressure and 25°C. The eluate was collected in a fraction collector with cuts made every 10 minutes. The first sample was obtained 30–45 minutes after the termination of irradiation.

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[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York. Some of it appears in more detail in a thesis by A. K. Bruce submitted in partial fulfillment of the requirements for the Ph.D. degree, Department of Radiation Biology, University of Rochester, 1956.

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The yeast was obtained from Standard Brands, Inc. and used from the center of the block. Its concentration was determined turbidimetrically, controlled by periodic dry weight determinations (Bruce, '56), and potassium analyses were done by flame photometry.

Irradiation. A yeast suspension of 10 mg/ml (wet weight) in distilled water was divided into two portions of 250 ml each, one being irradiated and the other, shielded by about 1 cm of lead, serving as a control. Each suspension was placed in a crystallizing dish giving a 20 mm depth of suspension and covered with a sheet of 1 mm thick lucite. Except where otherwise indicated oxygen, prewashed in a distilled water trap, was bubbled through the suspensions for 10 to 15 minutes before and continuously during the irradiation period.

Irradiation was carried out with a Picker Industrial x-ray unit operated at 250 kvp and 15 ma. Only inherent filtration was used and the center of the suspension was at a distance of 6 5/8 inches from the target. Dose rate determinations were made before each exposure using a 1000 r Victoreen Thimble chamber in place of the sample dish. All measurements were corrected for temperature and barometric pressure.[3] A dosage rate of about 1300 r/minute was obtained.

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[3] The irradiation and dose rate measurements were carried out by Mrs. Florence Van Slyke and Miss Patricia Ewart of the Radiation Tolerance Section.

The dose rate has not been corrected for backscatter which probably would result in an increase in total dose to the suspension of about 10%. The air dosage used was 60,000 r.

Test solutions. External substrates, where present, were 0.05 M. The temperature of elution was 25°C and the pH 3.5, the level ordinarily reached by a yeast suspension in distilled water, except for experiments with other ions present. In those cases, the pH was adjusted to 4.5 with HCl. Buffer was not desirable since it influences leakage rates and introduces extraneous ions. The pH was found to hold reasonably constant across the columns, changing by only 0.3 units, becoming more alkaline in the absence of substrate and more acid with glucose present. Other details will be presented with the experiments.

Manometric technique. For the tests of substrate utilization conditions were arranged to resemble as closely as possible those employed in the column, but the system was, of course, not strictly comparable. The ''direct'' method of Warburg was employed (Umbriet et al., '49) utilizing 2 ml of yeast suspension at 5 mg of yeast/ml when lactate, ethanol, and pyruvate was present, and 25 mg/ml when other substrates were tested. The substrates were tipped in from sidearms after temperature equilibration. Temperature was 25°C, shaking rate 120 strokes per minute. Initial pH was 3.5 adjusted with HCl, and measured both initially and terminally.

RESULTS

Effect of oxygen tension. For this experiment either oxygen *or* pre-purified nitrogen (less than 0.001% oxygen) was bubbled through the yeast suspension before and during irradiation. Other procedures were essentially unchanged. Typical results of potassium leakage measurements carried out aerobically at pH 3.5 after irradiation with 60,000 r are shown in figure 1. While irradiation of yeast in an oxygen atmosphere brings about a marked increase in potassium leakage, little effect is noticeable when irradiation took place in nitrogen.[4]

The effect seen after irradiation in oxygen is typical of that observed in the absence of external substrate and in line with that published in our preliminary report (Bair et al., '56). It is clear that the radiation effect on ionic transfer requires the presence of oxygen during irradiation, at least at the dose levels employed in these experiments.

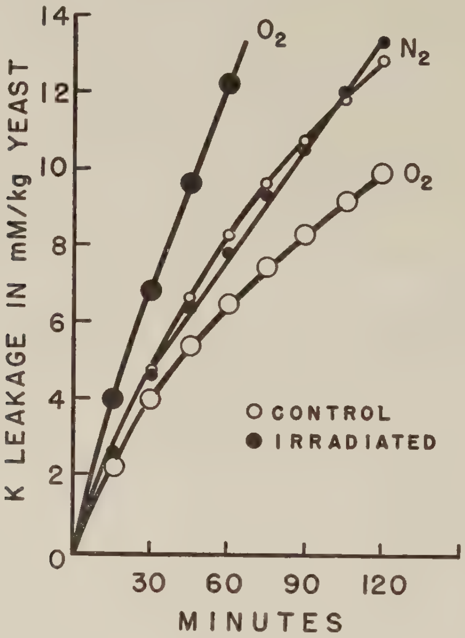

Fig. 1 Effect of gaseous environment during irradiation on endogenous *aerobic* potassium leakage from yeast after 60 Kr.

Effect of ionic environment. Since potassium loss by yeast is a cation exchange process, essentially by exchange for hydrogen ion (Rothstein and Bruce, '58b; Conway and Brady, '50; Rothstein and Enns, '49), it is, of course, possible that the radiation effect might be on the inward transfer of hydrogen ion, rather than the outward transfer of potassium ion. Direct measurement of this possibility could not be undertaken in the present experiments, but it was considered that the addition of other cations to the external solution could provide an indirect measure of the probable importance of this factor. For this purpose, a comparison of potassium leakage rates was made without added ions or with Li^+, Na^+, TEA^+ (triethylamine), or Cs^+ ions present in the eluting medium at 10^{-3} M, with pH adjusted to 4.5 with HCl. The determinations were made both in the presence and in the absence of external substrate.

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[4] The fact that the control leakage rates with nitrogen differ from those for the unirradiated yeast treated with oxygen during the "sham irradiation" period would seem to indicate that some metabolic alteration had occurred during the approximately one-hour period that the yeast was exposed to the gases. A consistent decrease in the rate of potassium leakage with the time of oxygen bubbling was observed. With nitrogen bubbling the rate remains the same.

The results are summarized in table 1. Rates of K^+ leakage were determined from the slopes of curves like those in figure

TABLE 1

Effect of ionic environment on potassium leakage at pH 4.5 and 25°C

ADDED ION	ENDOGENOUS			% NORMAL K^+ RETENTIVITY	GLUCOSE			% NORMAL K^+ RETENTIVITY
	K^+ leakage in mM/kg/hr				K^+ leakage in mM/kg/hr			
	Cont	Irrad 60 Kr	Δ		Cont	Irrad 60 Kr	Δ	
None	1.8	3.3	1.5	55	9.6	12.1	2.5	79
	1.2	3.0	1.8	40	12.3	13.6	1.3	90
	1.1	2.3	1.2	48				
	1.4	2.8	1.4	50				
Li^+	1.0	5.1	4.1	20	9.4	12.2	2.8	77
	2.5	6.5	4.0	38	10.1	10.7	0.6	94
	2.9	6.4	3.5	45	8.0	9.9	1.9	81
Na^+	2.5	4.3	1.8	58	6.4	6.4	0	100
	1.2	3.6	2.4	33	6.6	6.6	0	100
TEA^+	1.7	4.3	2.6	40	12.8	12.8	0	100
	2.9	4.4	1.5	66	16.0	16.0	0	100
Cs^+	3.9	7.7	3.8	50	11.5	11.5	0	100
	3.2	8.4	5.2	38	14.2	14.2	0	100

Initial concentration of ions 10^{-3} M.

Each line across represents a separate experiment.

1. In general, they were fairly constant with time, but if not, the initial rate was used.

Although substantial changes in leakage rates occur in the various ionic environments, the effect of irradiation, expressed as percent of the normal K^+ retentivity, remains about the same with the possible exception of Li^+. In fact, the most prominent feature of the influence of added external cations is the marked depression, in both control and irradiated yeast, of the K^+ leakage in the presence of Na^+ and glucose (mean control rate falls from 11.0 mM K^+/kg to 6.5 mM K^+/kg/hr. No such influence of Na^+ is seen in the absence of external substrate, where the effects of all cations seem to be a slight increase in leakage rate over that seen with H^+ alone in the external medium.

Thus, there is no evidence from these experiments that the radiation effect on K^+ transfer is markedly altered by providing other cations besides H^+ for exchange. Nor is the effect obviously otherwise dependent on ionic environment, despite marked changes in absolute rates of leakage.

Effect of external substrate. The experiments shown above (table 1), and those published in our preliminary report (Bair et al., '56), show that the rate of K^+ leakage is much higher in the presence of glucose than in its absence confirming previous work linking the rates of potassium leakage to metabolic rates (Rothstein and Bruce, '58b). Unexpectedly, the radiation effect is greater both in absolute and relative terms when exogenous substrate is *excluded.* In fact, only with H^+ or Li^+ does irradiation produce *any* decrease at all in potassium retentivity in the presence of glucose (table 1). To explain this relationship further the experiments with glucose were repeated and a number of other substrates exployed. For these the pH was adjusted to 3.5 since the rate of H^+ leakage is considerably higher and the slopes of the leakage rate curves more easily determined. This pH change results also in a difference in endogenous rates from those reported in table 1.

A typical experiment, done under the conditions just described, is shown in figure 2, using glycerol, pyruvate, or no added substrate. The results of a large number of such experiments are summarized in table 2. Clearly, a wide range of leakage rates was found as a function of the external substrate added. The highest rates occurred in the presence of the hexoses and trans-aconitate.[5] With other members of the glycolytic pathway the leakage rate was about one-half as rapid.

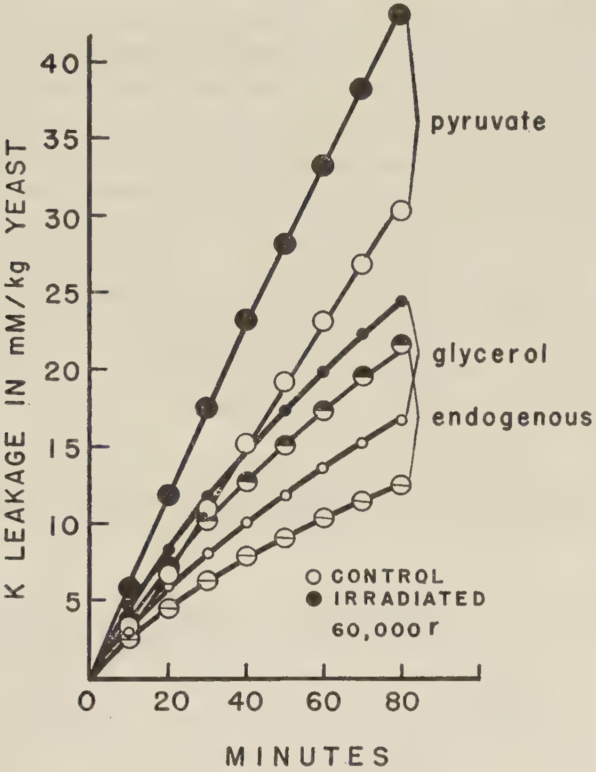

Fig. 2 Potassium efflux from yeast as influenced by various substrates. Closed or partly closed symbols refer to irradiated yeast in each case.

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[5] Note that trans-aconitate is not the physiologically active form of aconitic acid, and may be regarded as an inhibitor rather than a substrate.

The effects of irradiation on potassium leakage in the presence of the different substrates present an interesting picture (fig. 2 and table 2). The greatest measurable radiation effects, with the exception of those on endogenous metabolism, are seen when the three-carbon members of the glycolytic pathway are used as substrates. Of these, the greatest absolute effect is shown by pyruvate, although percentagewise, as given by the percent of normal potassium retentivity in the table, this is no greater than the others in this group. Little effect of irradiation is seen when measurements are made with the hexose sugars, glucose and mannose, but with fructose there was a greater potassium leakage rate in the irradiated than in the control cells. For both glucose and mannose the loss from irradiated and control yeast was essentially the same, while with glucose the rate tended to be slightly higher in the control, in contrast to all other substrates used. Of the members of the Krebs cycle tested, only citrate showed a radiation effect while succinate and trans-aconitate [5] did not.

Substrate utilization. The possibility of a correlation between potassium leakage, radiation effect, and substrate utilization was investigated by measuring O_2 uptake with each substrate.

The manometric measurements indicate that all substrates tested, with the exception of citrate and trans-aconitate, are metabolized by this yeast under aerobic conditions. The results of the several experiments are shown in table 3 expressed as µl of gas per mg wet weight of yeast. It may be noted that although both glycerol and citrate resulted in the consumption of more oxygen than did yeast without substrate; the difference was limited to the first hour in the case of citrate and practically so in the case of glycerol. The R.Q. values indicate incomplete oxidation in some instances, an excess of CO_2 production in others. It is interesting to note that trans-aconitate reduced both oxygen uptake and CO_2 production.[5] The pH was found to hold remarkably constant for most sub-

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[5] See footnote 5, page 331.

Effect of substrate on potassium leakage at pH 3.5 and 25° C

SUBSTRATE	K⁺ LEAKAGE IN mM/kg/hr.			% NORMAL K⁺ RETENTIVITY
	Cont	Irrad 60 Kr	Δ	
Endogenous	6.0	10.0	4.0	60
	6.5	10.1	3.6	64
	7.1	11.5	4.4	62
	5.0	7.5	2.5	67
	7.5	13.0	5.5	58
	5.0	9.0	4.0	56
	5.0	8.0	3.0	63
	6.0	8.0	2.0	75
	3.5	7.0	3.5	50
	4.7	7.8	3.1	60
	3.9	6.6	2.5	59
	3.0	7.0	4.0	43
Average	5.3	8.8	3.5	60
Glucose	34.5	33.5	— 1.0	103
	37.0	30.5	— 6.5	120
	28.5	25.5	— 3.0	110
	26.0	25.5	— 0.5	104
	31.7	31.7	0	100
	28.0	32.0	4.0	88
Average	30.8	29.8	— 1.0	103
Fructose	21.0	26.0	5.0	81
	22.0	23.5	1.5	94
	16.5	18.0	1.5	91
	16.0	21.0	5.0	76
	25.0	27.0	2.0	93
Average	20.1	23.0	2.9	87

SUBSTRATE	K⁺ LEAKAGE IN mM/kg/hr.			% NORMAL K⁺ RETENTIVITY
	Cont	Irrad 60 Kr	Δ	
Mannose	22.5	22.5	0	100
	23.5	23.5	0	100
Glycerol	11.0	16.0	5.0	69
	5.8	10.0	4.2	58
Pyruvate	25.5	34.5	9.0	74
	32.5	50.0	17.5	65
Ethanol	10.5	12.5	2.0	84
	14.0	16.5	2.5	85
Lactate	6.5	8.5	2.0	76
	13.0	16.0	3.2	81
Citrate	9.0	10.5	1.5	86
	12.5	17.6	5.1	71
	14.5	19.0	4.5	76
Aconitate	22.5	22.5	0	100
(trans-)	39.0	35.0	— 4.0	110
Succinate	15.5	15.5	0	100
	19.0	20.0	1.0	95

strates tested, measuring from 3.40 to 3.54 at the end of the experiment in all cases except glycerol when it rose to 3.9 and with endogenous substrate when it rose to 5.2. The possible significance of these results will be described below.

DISCUSSION

The present experiments provide clear evidence for an effect of ionizing radiation on the ability of the yeast cell to retain its intracellular potassium. They indicate that at this dosage level the damage takes place only when oxygen is present in the medium during irradiation. It appears to be independent of changes in the ionic environment during measurement, but its manifestation is markedly dependent on the presence or absence and the nature of the exogenous substrate. This latter aspect requires more detailed consideration.

The differences seen here in rates of potassium leakage elicited by various substrates capable of being utilized by the cell are not simply explained. The results indicate that only a very tenuous relationship, if any, exists between metabolic rate, as determined by oxygen consumption or carbon dioxide production, and the leakage rate of potassium from the unirradiated control yeast. In some instances high metabolic rates result in high leakage rates, while very low metabolic rates, as during endogenous metabolism or while utilizing glycerol, result in low rates of potassium leakage. However, there are many exceptions. Lactate and ethanol which are metabolized at a relatively high rate yield low potassium leakage rates, of the same order of magnitude as those seen with citrate which is not metabolized at all. Trans-aconitate,[5] which reduces metabolism below the endogenous level, brings about an unusually high potassium leakage rate. Thus, the nature of the energy source appears to play an important role, and each situation may present a separate problem. As pointed out by Rothstein and Bruce ('58b), the redox poten-

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[5] See footnote 5, page 331.

Effect of substrate upon the respiration of yeast at pH 3.5 and 25°C, air in gas space

SUBSTRATE	GAS EXCHANGE IN μL/MG				R.Q.		
	O_2 Consumption		CO_2 Production		60′	120′	Theory [1]
	60′	120′	60′	120′			
Glucose	15.82	31.30	21.42	37.27	1.35	1.19	1.00
	15.12	29.97	19.43	35.65	1.29	1.19	
	14.18	28.36	16.08	30.42	1.13	1.08	
Fructose	16.09	29.76	15.46	29.53	1.10	1.07	1.00
	15.31	28.25					
	14.10	27.53					
	14.26	27.57					
Mannose	15.43	28.20	17.75	32.62	1.15	1.16	1.00
	15.57	28.78					
	15.00	29.76					
Glycerol	3.20	4.89	2.61	3.93	0.82	0.80	0.86
	3.12	4.81	2.64	4.06	0.85	0.84	
Pyruvate	8.06	15.75	8.28	16.22	1.03	1.03	1.20
	7.52	15.56					
Ethanol	14.54	26.85	5.76	11.12	0.40	0.41	0.66
	14.34	26.61	5.93	11.18	0.41	0.42	
Lactate	13.50	25.93	14.13	26.72	1.05	1.03	1.00
	13.86	26.52	14.25	27.34	1.03	1.03	
Citrate	3.20	4.42	2.93	4.12	0.92	0.93	1.33
	3.04	4.22	2.46	3.58	0.81	0.85	
Aconitate (trans-)	1.61	1.82	1.64	2.09	1.02	1.15	1.33
	1.41	1.64	1.83	2.50	1.30	1.52	
Succinate	4.12	7.11	4.25	7.67	1.03	1.08	1.14
	3.88	6.57	3.96	7.13	1.02	1.08	
Endogenous	2.74	3.98	2.71	3.91	0.99	0.98	1.00
	2.53	3.66	2.35	3.36	0.93	0.98	

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[1] On the basis of complete oxidation of the substrate. Values greater than the theoretical indicate the amount of aerobic fermentation taking place.

tial may be a more important parameter than metabolic rate *per se.*[6]

The relationship between either metabolic rate or absolute leakage rate and the radiation-elicited increase in leakage rate is summarized in figures 3 and 4. In figure 3, it is seen that the percent of normal potassium retentivity after irradiation might be considered in a general way to be higher with substrates which are rapidly metabolized. However, the relationship is not consistent quantitatively, and even if one considers trans-aconitate as an inhibitor and therefore in a different category, marked exceptions are seen with citrate and succinate.

If the radiation effect is plotted against normal potassium leakage rate, a somewhat clearer picture appears, as seen in figure 4. Here only pyruvate is badly out of line in showing both a marked radiation effect and a high normal leakage rate. Otherwise the trend is for a greater radiation effect — on a percentage basis — at the lower leakage rates. Thus, in general, the substrates which are associated with lower leakage rates result in a greater radiation effect as measured by percentage of normal potassium retentivity.

Consideration of the radiation effect in absolute terms does not eliminate the differences noted (cf. table 2). It will be seen, however, that the change (column $\triangle$ in table 2) tends to be either zero, negative, or of the order of 2 to 5 mM K^+/kg/hr. Pyruvate is again an exception. Since a single dose, 60 Kr, is involved in all cases, it might be concluded that damage, if it appears at all, is of the same order, in terms of absolute increases in leakage, with all substrates. The lack of effect with some substrates may indicate that certain reactions are more closely associated with the transfer mechanism than others. However, the fact that a radiation effect occurs with

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[6] The values shown in table 2 for the various substrates tested are somewhat different from those reported by Rothstein and Bruce ('58b). The only difference between their experiments and ours is the more extended starvation period in the radiation experiments. Control experiments show that this factor accounts for the difference seen.

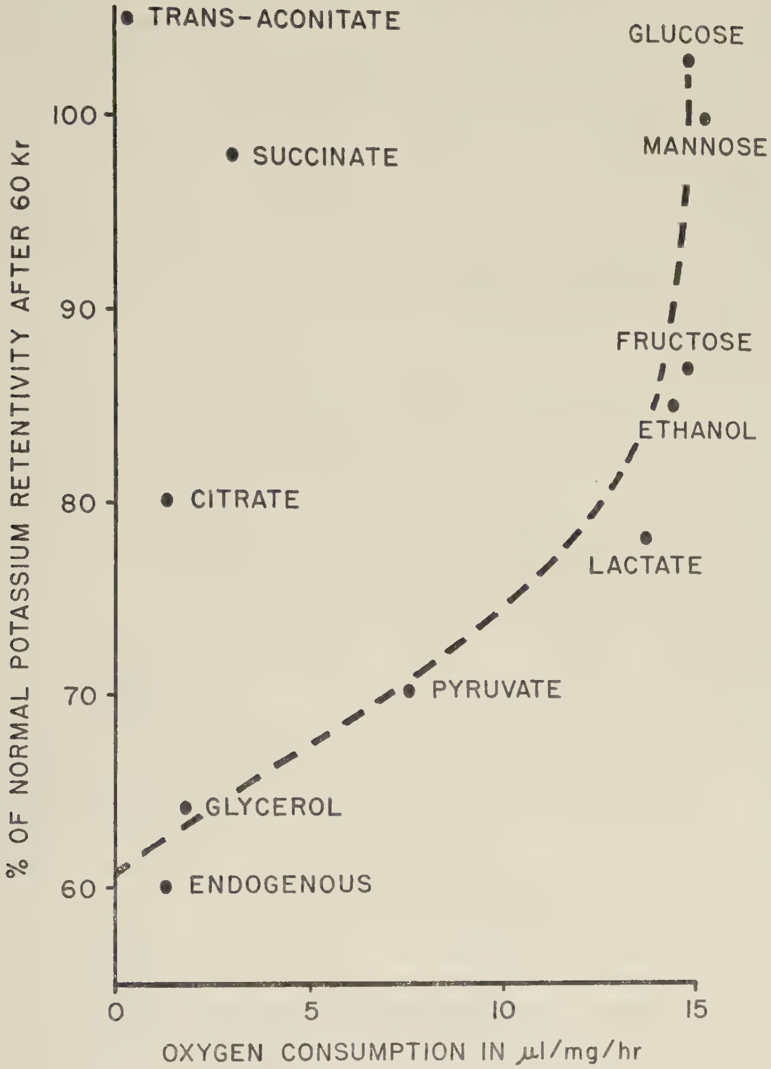

Fig. 3 Radiation effect as a function of oxygen consumption rate with various substrates.

fructose and not with glucose or mannose indicates the extreme specificity that must be involved if this is true. All these sugars are phosphorylated by hexokinase at the cell surface (Kunitz and McDonald, '46, and Berger et al., '46)

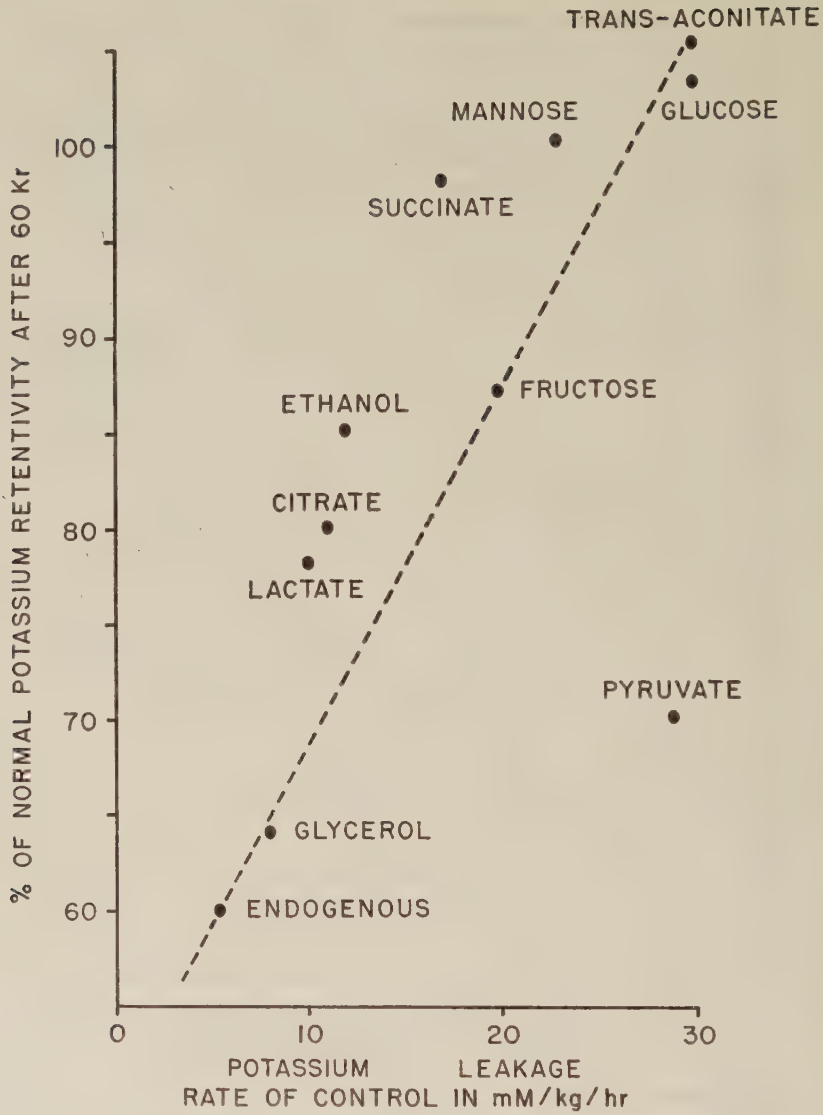

Fig. 4 Radiation effect as a function of the potassium leakage rate of unirradiated yeast with various substrates.

and enter readily into cellular metabolism. Also, the results with citrate must be explained. A direct effect on the membrane may occur in this case, and with trans-aconitate.

The lack of a more definite agreement between metabolism, as measured by oxygen consumption, and the magnitude of the radiation effect may in part be due to the properties of the system measured. Measurements of oxygen consumption result in values which are representative of the over-all metabolic activity of the whole cell. What may be of more importance here are the reactions at or near the sites of potassium exchange, which presumably could be quite different in activity from those representative of the whole cell.

SUMMARY

1. Leakage of potassium from yeast receiving 60,000 r of x-irradiation has been studied by a method analogous to an ion exchange column.

2. Irradiation with 60 Kr produces a marked disturbance in the ability of the yeast cell to retain potassium ion.

3. The effect is dependent on the presence of oxygen during irradiation, but is not markedly altered by changes in ionic environment during the measurement period.

4. The manifestation of the radiation effect is very dependent on the substrate present during measurement. In a general way, when substrates associated with high normal leakage rates are used the radiation effect is small and vice versa, with pyruvate a notable exception. There was a fairly linear inverse relationship between normal leakage rate and the magnitude of the radiation effect.

5. Correlation between substrate utilization, as measured by oxygen uptake, and the degree of radiation damage manifested was not as good as that with normal potassium leakage rates.

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THE ENERGY REQUIREMENTS OF MELANIN GRANULE AGGREGATION AND DISPERSION IN THE MELANOPHORES OF ANOLIS CAROLINENSIS [1]

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FIVE FIGURES

It has long been known that the rapid color changes of the skin of the various poikilothermic vertebrates is, in part, a function of the activity of the melanin pigment-bearing cells, the melanophores. The darkening and lightening of melanophores is due entirely to intracellular pigment movement. The lightening (contraction) of melanophores is due to the aggregation of melanin granules in the cell's perikaryon; while darkening (expansion) is due to the dispersion of the pigment granules to the periphery of the cell, the dendritic processes. This entire lightening and darkening cycle apparently takes place without appreciable change in the shape or dimension of the cell.

Somewhere within the cycle of the aggregation and dispersion of melanin granules energy must be expended. The question of whether this energy is utilized by the melanophore to produce expansion, contraction, or some intermediate state, has been recurrently investigated over a century (Parker, '48). Recently this question has been reinvestigated in isolated frog skin, particularly by the use of metabolic inhibitors

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[1] This investigation was supported in part by grants from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and by the United States Public Health Service Grant #RG–2912 (C4).

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and the energy-rich intermediate adenosine triphosphate (ATP).

Wright ('52, '53 and '55) has utilized such metabolically active agents as cyanide, azide, iodoacetate, malonate, fluoracetate, and triphenyltetrazolium. His conclusion, though admittedly tentative, is that energy is required for pigment movement in general, whether it be aggregation or dispersion. In unexplained contrast to this point of view is Wright's belief that the melanophore expanding hormone (MSH) acts to darken melanophores by inhibiting glycolysis.

Lerner and Takahashi ('56) utilizing essentially identical tissue preparations as Wright disagree with Wright's findings. They suggest that energy provided by ATP is used for darkening melanophores and that MSH acts directly or indirectly to "activate" ATP. These conclusions are based on the observations that ATP will cause the dispersion of melanin in the melanophores of isolated frog skin;[3] that pretreating isolated frog skin with 2,4-dinitrophenol will inhibit darkening on MSH; that inhibition (presumably of cytochrome oxidase) with carbon monoxide also prevents darkening on MSH and that melanophores cannot be darkened by MSH in an oxygen-free environment. Unfortunately, Lerner and Takahashi's work is not accompanied by any quantitative data.

In view of the conflicting opinions held by investigators working on isolated frog skin it seems profitable to report on our results with *Anolis carolinensis*. Skin isolates from this lizard have been found to possess a number of advantages over the fundulus scale and frog skin preparations commonly used in studies in melanophore physiology.

The skin of *Anolis* may be removed from the animal with ease and floated on Ringer solution. The surface of the skin appears smooth to the eye, though it does, in fact, consist of many minute scales. Almost the entire dorsal skin is homogeneous in response and color. All pieces used in an experiment can be taken from a single animal and are thus genetically identical. The skin is thin but tough and lacks blood vessels. This indicates that its nutrients and oxygen normally diffuse in from underlying tissue, and waste products diffuse out. Therefore, the situation in which the skin is floated on Ringer solution is physiologically similar to that of skin *in situ.*[4] This fact, and the fact that the behavior of *Anolis* melanophores is subject to easy quantification, makes this system uniquely suited for study.

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[3] Wright (in conversation) has been unable to confirm this observation.

When the melanophores of the skin of *Anolis* are fully expanded the lizard appears dark brown; when they are contracted the lizard appears bright green. If the melanophores are in some intermediate condition the skin appears as some hue of green, yellow, or brown. This metachrosis is due entirely to the distribution of the melanin granules, within the melanophore dendrites, relative to certain light reflecting and absorbing layers in the skin (von Geldern, '21; Fox, '53).

The nature of the hormonal control of *Anolis* color change was determined by Kleinholz ('38a, '38b). He found that in the intact lizard the darkened condition of the melanophores depends upon the presence of MSH.[5] In hypophysectomized lizards the melanophores are contracted but can be expanded through the injection of pituitary extracts. This response pattern was found to be reproducible in isolated skin floated on Ringer solution. On hormone-free Ringer solution the skin is bright green; when the skin is floated on Ringer solution containing MSH it turns brown; and with the removal of MSH it returns to its green color. Thus, in the normal economy of the lizard *Anolis,* the lightened condition of the skin is due to the absence of the hormone MSH in the blood of the animal, the darkened condition to its presence. Epinephrine is also implicated in the control of melanophore color, in stress conditions its presence in the blood leads to the rapid lightening of darkened melanophores.

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[4] Such is not the case in isolated preparations of either frog skin or fundulus scale.

[5] In the Kleinholz studies, and until 1953, this hormone was commonly called intermedin, due to its origin in most vertebrates in the pars intermedia of the pituitary.

MATERIALS AND METHODS

The animals used in these experiments were obtained from a dealer in Louisiana. They were kept in 3′ × 4′ × 4′ copper screen cages, watered daily and fed weekly on the larvae of *Tenebrio molitor*. The lizards were sacrificed by decapitation.

Excoriation is a simple matter: it is performed by cutting off the lizard's extremities, making a lengthwise median abdominal incision, slitting the skin around the base of the tail and the neck, and peeling the skin off the entire torso. Skin that is removed in this way separates easily from the underlying loose connective tissue. There is no bleeding. Because the skin is hydrophilic beneath and hydrophobic above, it floats on and adheres closely to the surface of Ringer solution.

After the skin had been removed from the lizard, it was floated on Ringer solution for two hours to allow it to become relatively free of endogenous hormone. The color of skin so treated is bright green. Skin in this condition is ready for use. It is then cut into pieces, conveniently 6 or 8 from a single skin, and the pieces floated on test solutions.

There were two parts to the experimental routine. In the first part, the effect of the treatment in question on melanophore behavior was noted visually and recorded. Various titers and combinations of agents were tested and each test was replicated two, three, or more times. In the second part, the effect of the treatment in question on melanophore behavior was recorded photometrically. In all tests, adjacent pieces of skin from the same animal were used for the control.

The photoelectric apparatus designed for the measurement of changes in reflectance of *Anolis* skin is described below. It measures the amount of light from a constant source, reflected from the surface of a piece of isolated lizard skin as it floats on a test solution. In the operation of the apparatus, the skin was so mounted as to be free on three sides when floated. The skin and mounting were placed in the center of a black glass container with a capacity of about 10 ml of fluid. (This glass reservoir could be exhausted by an aspirator tube and filled through two inlet tubes.) Skin properly placed was in a position so that part of the light reflected from its surface passed vertically through a brass tube of 3 mm diameter that was mounted in a microscope tube from which the lens had been removed. This light fell on a small area of a 931A photomultiplier tube in the search unit of Photovolt model 520M multiplier photometer. The constant light source was a GE 1493 tungsten bulb in a permanently mounted Spencer Microscope Lamp. Line current to the light source was kept constant by a Sola 30801 constant voltage transformer with an output of 6.0 volts. To prevent distortion due to difference in chromatic sensitivity of the photocell, the incident light was passed through a blue filter with maximum transmission at about 4600 Å.

As reflectance from the lizard skin changed, more or less light fell on the photomultiplier tube and this was recorded from the photometer.

The photoelectric apparatus was kept in a constant temperature room at 22°C. All solutions were permitted to come to room temperature before they were used. The temperature of the solution within the reservoir was recorded by means of a permanently mounted thermistor (Victory Engineering Corporation model 32A1). In the course of 9 months the temperature of test solutions varied no more than one degree on either side of 22°C.

All data are presented as a percentage of the reflectance of untreated skin: skin which had been floated on Ringer solution for at least two hours before being used.

All experiments were done with agents freshly dissolved in a Ringer solution which consisted of 6.50 gm NaCl, 0.25 gm KCl, 0.30 gm $CaCl_2$ per liter of distilled water. This solution was brought to pH 7.4 with 0.1N NaOH, and then buffered by adding 0.02 gm of NaH_2PO_4 per liter and sufficient Na_2HPO_4 in Ringer solution to give pH of 7.4. If this solution is changed every 12 hours or so, isolated skin floated on it will continue to respond to agents for at least 48 hours. In these experiments, however, skin was always used between two and 6 hours after excoriation.

The pituitary extract used in this study was made available by Dr. Irby Bunding, of Armour Laboratories, Chicago. It is lot #D216-155C and is an extract of the hog pituitary. Its MSH potency is, roughly, 1.4 × 10^8 units/gm as determined by Drs. Shizume and Lerner, according to their method (Shizume, Lerner, and Fitzpatrick, '54).

The source of the other agents used were as follows: epinephrine, as Adrenalin chloride, from Parke, Davis and Company; adenosine triphosphate (di Na salt) from Nutritional Biochemical Corporation; adenosine-5-phosphoric acid from Armour and Company; and 2,4-dinitrophenol, purified by repeated recrystallizations from hot water, was obtained through the courtesy of Dr. G. Williams-Ashman.

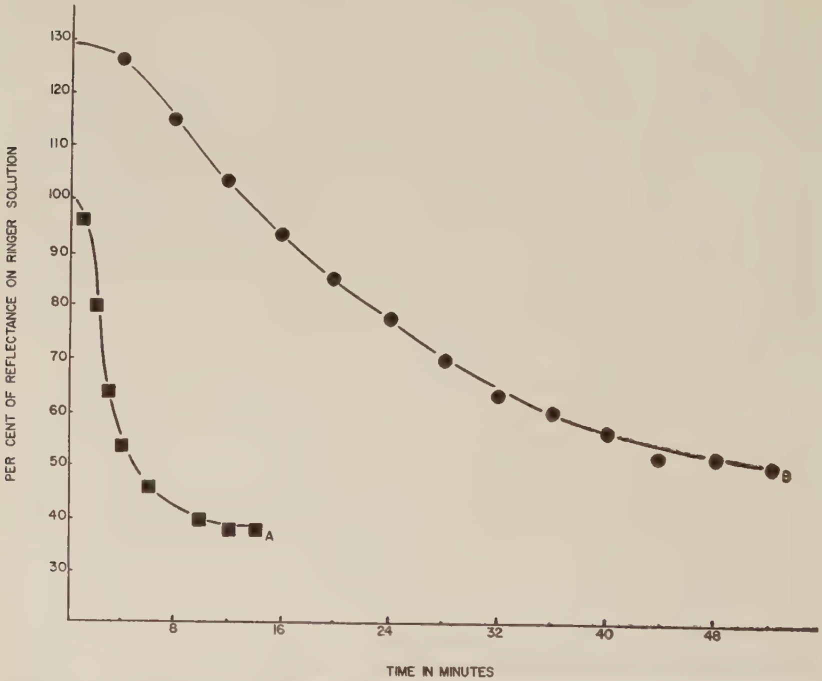

Fig. 1 The dispersion of pigment within the melanophores of *Anolis carolinensis* skin floated on MSH. A. Skin floated on Ringer solution containing 1.4 × 10^{-7} gm/ml of MSH rich pituitary extract. B. Skin floated on 1.4 × 10^{-7} gm/ml pituitary extract following treatment with 10^{-3} M ATP (di Na salt) for 20 minutes.

RESULTS

Hormones

When isolated skin, which has been permitted to turn bright green by floating on Ringer solution, is placed on a solution of pituitary extract there occurs a rapid darkening (fig. 1A). As is true in isolated frog skin (Shizume, Lerner and Fitzpatrick, '54), this darkening deepens, within the physiological range, in proportion to the concentration of the extract (fig. 2).

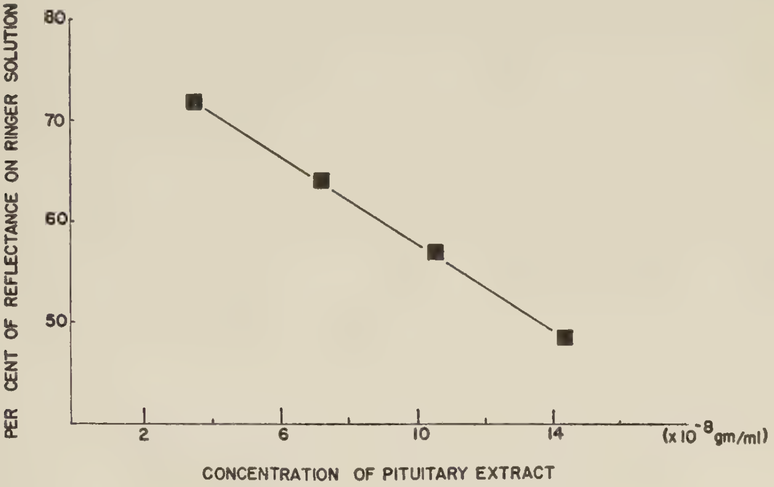

Fig. 2 The effect of MSH titer on degree of darkening of the skin of *Anolis carolinensis*. In this experiment adjacent pieces of skin from the dorsal surface of a single lizard were tested simultaneously on different titers of a weak solution (within the normal physiological range) of MSH rich pituitary extract. Reflectance is taken as the lowest value achieved during 30 minutes exposure to MSH.

Darkening on MSH is fully reversible if the skin is returned to Ringer solution (fig. 3A). Lightening is much slower than darkening.

If skin which has been darkened on MSH is placed on a solution of epinephrine, lightening is greatly enhanced (fig. 3B). The skin returns to its original reflectance (on Ringer solution) in about one-sixth of the time that would be required in the absence of MSH alone. Furthermore, when skin which has turned bright green on Ringer solution is placed on epinephrine the skin lightens to a "superlightened" condition (fig. 3C) and reflects more light than does skin in the absence of MSH alone.[6] This superlightening effect has been described in the isolated frog skin by Wright ('47, '55).

Nucleotides

Adenosine triphosphate. Lerner and Takahashi ('56) report that ATP without any other agent causes the dispersion of melanin granules in the melanophores of isolated frog skin and offered this as evidence that energy is used in darkening melanophores. In our experiments ATP had a diametrically opposite action. When skin which has turned bright green on Ringer solution is placed on ATP, the skin lightens to the superlightened condition (fig. 3D). Furthermore, when skin which has been darkened on MSH is placed on a solution of ATP, lightening is greatly enhanced (fig. 3E). If skin which has been superlightened by treatment with ATP is floated on MSH, the ensuing darkening is markedly inhibited (fig. 1B).

It was Lerner and Takahashi's assumption that the action of ATP was assignable to the energy yielding reactions of this compound. That this assumption cannot be accepted *a priori* has been made clear by contributions from muscle physiology, for while it is known that ATP can induce contraction of isolated, intact muscle cells, this contraction can also be induced with inorganic polyphosphates (Buchthal, Deutsch,

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[6] The action of epinephrine on *Anolis* melanophores is not typical of epinephrine action on melanophores in general. Though most of the skin is rapidly lightened by epinephrine, there are always a number of melanophores which respond to epinephrine by darkening (Kleinholz, '38b). This effect does not occur when vasopressors related to epinephrine are used; tyramine and hydroxytyramine both give rapid, even lightening (unpublished observations).

The atypical response of *Anolis* skin to epinephrine makes it difficult to obtain a true photometric record of the lightening phenomena, inasmuch as the photometric record includes some cells that are darkening with the many that are lightening. Furthermore, since the darkening is a less rapid event (with epinephrine) than lightening, there is generally a slight reversal in the photometric record after the skin has been on epinephrine for 8 to 10 minutes; this reversal is excluded from figures 3B and 3C.

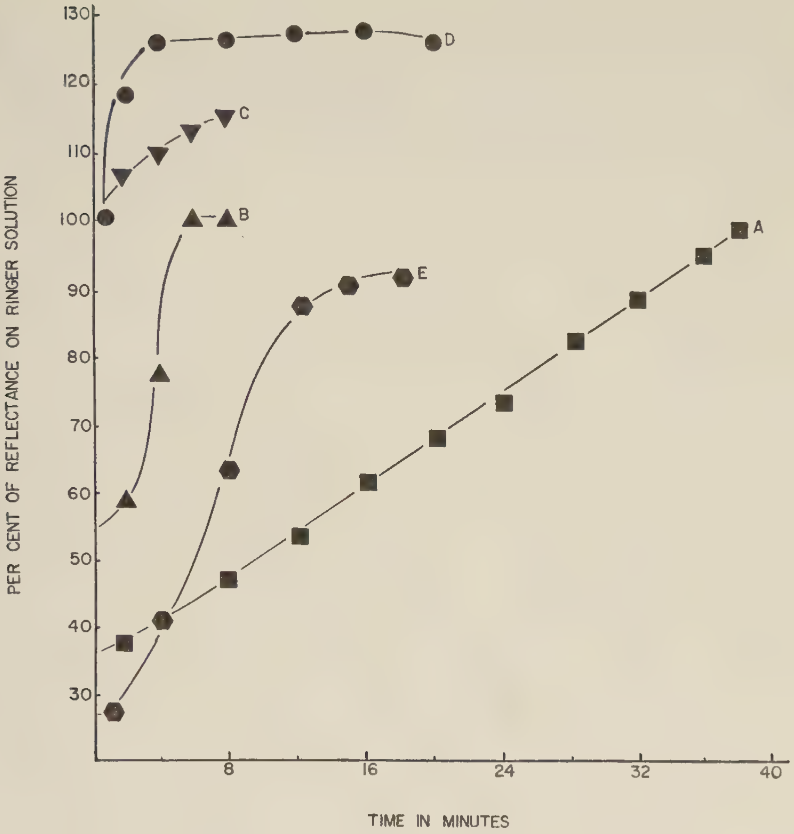

Fig. 3 The aggregation of pigment within the melanophores of *Anolis carolinensis* skin. A. Skin floated on Ringer solution following treatment with 1.4 × 10^{-7} gm/ml MSH rich pituitary extract for 10 minutes. B. Skin floated on 10^{-7} M epinephrine following treatment with 1.4 × 10^{-7} gm/ml pituitary extract for 10 minutes. C. Skin floated on 10^{-7} M epinephrine. D. Skin floated on 10^{-3} M ATP (di Na salt). E. Skin floated on 10^{-3} M ATP (di Na salt) following treatment with 1.4 × 10^{-7} gm/ml pituitary extract for 10 minutes.

and Knappeis, '44). Furthermore, ATP applied extracellularly to muscle apparently does not permeate the cell membrane (Boyle and Conway, '41) and cannot, therefore, serve as an energy source for contractile proteins within the cell. Finally, when ATP is injected directly into single muscle fibers it produces neither potential changes or contracture, though both of these are produced by external application

(Falk and Gerard, '54). The mode of ATP action on intact muscle has recently been demonstrated by Falk ('56) to be attributable to the ability of ATP to bind Ca^{++} ions in Ringer solution. Such Ringer solution, in which Ca^{++} is chelated, cannot support the cell membrane in the polarized condition. To test whether the lightening action of ATP could be attributed to its ability to bind Ca^{++}, skin which was darkened on MSH was then lightened on Ca^{++} deficient Ringer solution (fig. 4).

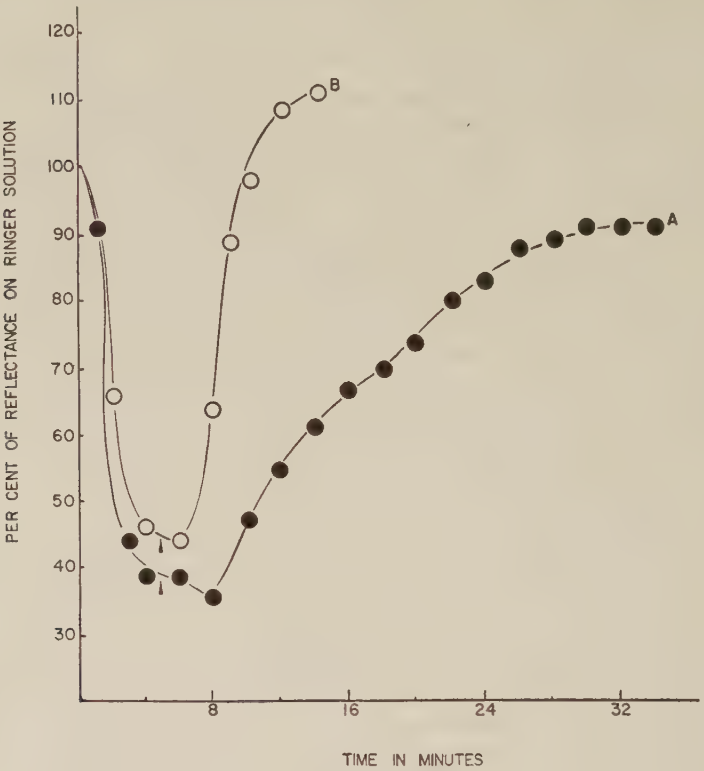

Fig. 4 The effect of calcium deficiency on pigment aggregation in *Anolis* melanophores. A. Skin floated on calcium-deficient Ringer solution containing 5.6 × 10^{-7} gm/ml of MSH rich pituitary extract for 5 minutes (wedge) at which time it was floated on the regular Ringer solution. B. Skin floated on calcium deficient Ringer solution containing 5.6 × 10^{-7} gm/ml of MSH rich pituitary extract for 5 minutes (wedge) at which time it was floated on the calcium-deficient Ringer solution devoid of pituitary extract.

It was found that though darkening by high concentrations of MSH could proceed in this Ca^{++} deficient Ringer, lightening was greatly enhanced by such solutions.

Adenosine monophosphate. It seemed improbable that the behavior of ATP in causing the darkening of melanophores in frog skin and their lightening in lizard skin could be traced to a single action. It is known that the xanthine caffeine will darken melanophores (Wyman, '24; Lerner and Takashashi, '56). As this compound is related to the purine degradation products of ATP we investigated the possibility that adenosine-5-phosphoric acid (AMP), the nucleotide degradation

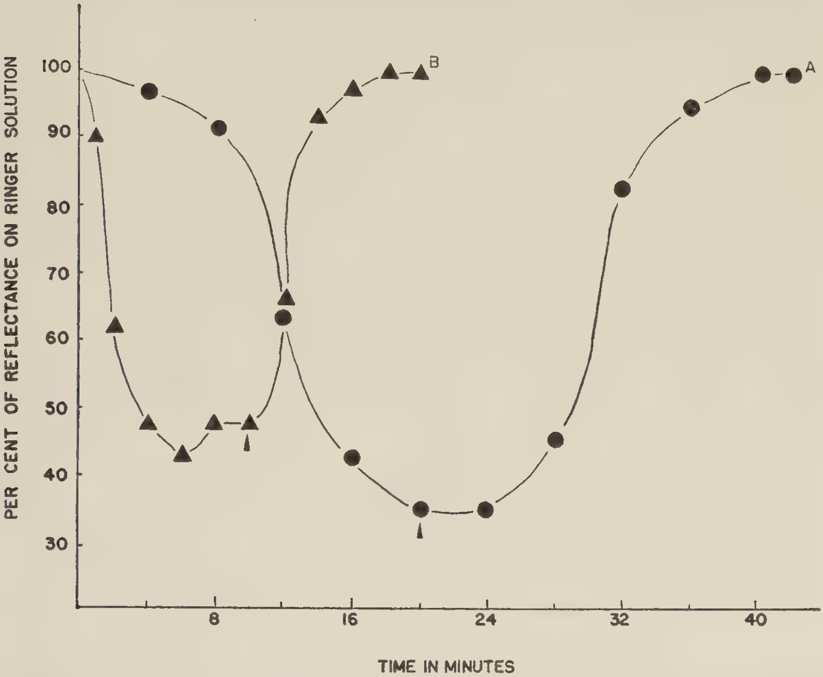

Fig. 5 The reversible dispersion of pigment within the melanophores of *Anolis carolinensis* skin treated with adenosine-5-phosphoric acid and 2,4-dinitrophenol. A. Skin floated on 10^{-2} M adenosine-5-phosphoric acid (AMP) at pH 7.4 for 20 minutes (wedge) at which time it was floated on Ringer solution. B. Skin floated on 10^{-4} M 2,4-dinitrophenol for 10 minutes (wedge) at which time it was floated on Ringer solution.

product of ATP, was capable of causing dispersion of pigment within melanophores. To test this possibility, skin which had been permitted to turn bright green on Ringer solution was placed on AMP dissolved in Ringer solution. It was found that AMP is capable of producing profound darkening of lizard skin and that this darkening is fully reversible on Ringer solution (fig. 5A).

Metabolic inhibitors

It seems evident from the foregoing observations that the direct treatment of melanophores with ATP divulges nothing about the energy requirements of the intracellular aggregation and dispersion of melanin granules. The less direct approach of reducing the energy available for pigment movement with metabolic inhibitors may be more helpful. Wright ('52, '53, and '55) has used cyanide, azide, and fluoracetate and found them without effect on melanophores. He found iodoacetate substantially decreases the ability of melanophores to lighten, following darkening on MSH, and construed this to mean that energy is required for melanin pigment aggregation. Our experience with iodoacetate has confirmed Wright's findings to the extent that we find that while iodoacetate has only a limited ability to darken lizard skin it inhibits the lightening of skin following darkening on MSH. Also, iodoacetate acts synergically with MSH to cause intense darkening of lizard skin with weak hormone solutions. However, we have found that sulfhydryl inhibitors in general have a complex effect on melanin dispersion and convergence (Horowitz, '56 and '57) which cannot be attributed entirely to effects on glycolysis. Therefore, the action of iodoacetate may be on –SH groups other than those concerned with glycolysis.

Unfortunately, there is no agent which will selectively inhibit the synthesis of ATP by all metabolic pathways, nor any which will inhibit enzymes with ATP as their substrate to the exclusion of all others. However, 2,4-dinitrophenol (DNP) is capable of the quite specific blockade of oxidative phosphorylation (Loomis and Lipmann, '48) by an incompletely understood process whereby ATP synthesis is "uncoupled" from most respiratory processes. It was thought to be of interest to determine the effect of DNP on pigment aggregation and dispersion.

When green skin is floated on a solution of DNP, immediate darkening takes place (fig. 5B). This darkening will occur at titers as low as 5×10^{-5} M, and less completely, at 10^{-5} M. If skin darkened on DNP is returned to Ringer solution, a complete recovery ensues, the skin returning to its bright green color.

Two other uncoupling agents were tested (Judah and Williams-Ashman, '51), Pyocyanin dihydrochloride and Janus green B. Pyocyanin fully darkened skin at dilutions of one part in 1000 but only slightly at one part in 10,000 of Ringer solution. Janus green B darkened skin at 10^{-3} M but not appreciably at 10^{-4} M. Darkening on Pyocyanin is reversible on Ringer solution, that of Janus green B is not.

DISCUSSION

The action of epinephrine and MSH on the melanophores of isolated skin is one of the oldest known of the few *in vitro* systems in which there is no doubt that the *in vivo* hormone action is reproduced. The system has some potential from the point of view of the study of the mechanism of hormone action but is severely limited in this respect by the impossibility, at present, of separating chemical changes in the melanophores proper from the changes in the mass of surrounding tissue. For this reason, care is essential in attempting to interpret the action of agents which elicit changes. There is no check as to whether the anticipated action of the agent, which led to its use, is in fact the action responsible for whatever may be the effect on melanin granule movements.

Thus, though the point is not proven, it seems extremely unlikely that the effect of ATP observed by either Lerner and Takahashi ('56) or ourselves is attributable to whatever energy yielding potentialities this compound may have. In the case of ATP action on *Anolis* skin, the lightening effect seems attributable to ATP's ability to bind Ca^{++} in the Ringer solution and at cell surfaces. In the case of the darkening action of ATP on frog skin melanophores, we are probably dealing with a non-specific effect associated with the adenosine structure of ATP. The fact that frog melanophores are deeply imbedded in tissue may explain their apparent immunity from the Ca^{++} binding activity of ATP.

The energy requirements of dispersion and aggregation are still to be resolved. Lerner and Takahashi ('56) believe that pigment dispersion is the important energy consuming part of the cycle. They report that if a piece of frog skin is "made to darken and lighten by immersion in Ringer's solution with and without MSH, after several hours the melanocytes become fatigued and failed to respond to MSH. After ATP was added to the fatigued skin, MSH was enabled to cause dispersion of the granules again." However, they also claim that ATP "without any other agent caused dispersion of melanin granules in isolated pieces of frog skin." Lacking quantitative data, we cannot hope to separate the darkening due to ATP from that due to MSH, but, in any case, it is evident from the observations above that there is substantial cause to doubt that ATP is acting to contribute energy to the system. Furthermore, Wright ('55) alternately treated isolated frog skin with pituitary extract and Ringer solution while simultaneously poisoning the skins with cyanide or azide. He found no reduction in either the dispersion or aggregation response over 4 hours thus contradicting Lerner and Takahashi's observations (under even more rigorous experimental conditions). Wright found that if iodoacetate is used in this type of experiment the melanophores become progressively less able to lighten both on hormone-free Ringer solution and on epinephrine solutions. Sodium fluoride has an effect similar to that of iodoacetate. We have been able to confirm Wright's observations on iodoacetate on lizard skin melanophores.

Lerner and Takahashi ('56) found that pretreating frog skin with carbon monoxide prevents the darkening action of

MSH and that this inhibition is, in part, released by light. Furthermore, they found that the dispersion of melanin granules is inhibited by an atmosphere of hydrogen or helium and that this inhibition is reversed by oxygen. It seems remarkable that this be the case since Lerner, Shizume and Bunding ('54) found that MSH darkening proceeds well under high vacuum. Assuming, however, that oxygen is necessary for MSH action, both Lerner and Takahashi's ('56) and Wright's ('55) observations on what appears to be the considerable energy reserves of melanophores indicate that anaerobic inhibition of MSH action does not act through energy yielding processes.

Lerner and Takahashi ('56) found that 2,4-dinitrophenol, while incapable itself of changing the degree of dispersion of a frog skin melanophore, prevents the darkening action of MSH. Unfortunately, they do not report on the effect of DNP on already darkened skin. In our experiments we found that DNP rapidly darkens skin in a completely reversible reaction and at titers consistent with those associated with its ability to uncouple oxidative phosphorylation. Furthermore, other uncoupling agents were found to have similar effects. We cannot reconcile these observations with Lerner and Takahashi's except to again suggest that their results may reflect the poorer permeability of the frog skin preparations.

It may be of some significance relative to the question of energy requirements of darkening and lightening that in our experience whenever lizard skin was permitted to die without the benefit of fixation, the melanophores were always in the expanded condition. Conversely, we have never seen a lizard skin which was irreversibly lightened.

Lerner and Takahashi's conclusions notwithstanding, it seems likely from the available evidence that it is during the period in which the melanin granules are aggregating and aggregated that the most metabolic energy is utilized in the melanophore aggregation-dispersion cycle. The removal of the sources of metabolic energy results in dispersion of the melanin granules. However, we would caution that this should not be construed as proof or even substantial evidence for the current idea (Wright, '55 and Lerner and Takahashi, '56) that MSH acts by influencing the availability of metabolic energy in the cell.

SUMMARY

1. The isolated skin of the lizard *Anolis carolinensis* is reintroduced as a tool in the study of melanophore physiology and hormone action. Its advantages and disadvantages relative to existing systems are briefly reviewed.

2. A device for making continuous quantitative measurements of the effect of agents on melanin granule dispersion and aggregation is described.

3. The melanocyte stimulating hormone (MSH), adenosine monophosphate, 2,4-dinitrophenol and other uncoupling agents favor pigment granule dispersion in melanophores.

4. Adenosine triphosphate, calcium deficient Ringer solution, and epinephrine favor the aggregation of pigment within melanophores.

5. The significance of these observations to current ideas in melanophore physiology are discussed.

The author wishes to express appreciation for the advice, assistance, encouragement and friendship of Dr. Dorothy Price and to gratefully acknowledge the advice and assistance of Dr. H. G. Williams-Ashman and Dr. Hewson H. Swift. This work was done while a John M. Prather Research Fellow of the University of Chicago and a National Science Foundation Predoctoral Fellow.

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THE EFFECT OF LUMINAL ON GROWTH, GLUCOSE UTILIZATION AND OXYGEN CONSUMPTION IN MAMMALIAN CELLS IN TISSUE CULTURE

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FIVE FIGURES

INTRODUCTION

The way in which growth, that is the formation of new protoplasm and new organisms, takes place continues to be one of the basic problems in biology. In attempts to elucidate the various factors concerned with the initiation, regulation and cessation of growth many and varied approaches have been used. Numerous investigations have been concerned with the chemical machinery whereby energy is made available for growth processes. In these studies use is often made of substances which inhibit the energy releasing series of reactions in an effort to learn something about any relationship existing between the production of energy and synthetic processes. The present study, which is of this kind, deals with the effect of luminal, one in a class of imperfectly defined substances known as narcotics, hypnotics and anaesthetics. These compounds are generally believed to exert their effects by interfering with the activity of one or more of the many enzymes functioning in the cell. Their action does not appear to involve a very specific type of reaction with a particular enzyme so that narcotics, hypnotics and anaesthetics are frequently regarded as indifferent inhibitors—to distinguish them from such substances as cyanide, for example, which is known to inhibit specifically by combination with enzymes which contain heavy metals.

Among the earliest experiments comparing the inhibitory action of narcotics on respiration and cell division were those of Loeb and Wasteneys ('13). These authors showed that all division in sea-urchin eggs could be stopped by concentrations of various narcotics which, at most, removed only 20% of the normal oxygen consumption. Similar results have since been reported by a number of other investigators such as Clowes and Krahl ('40), Moog ('44) and Burt ('45). It has been emphasized elsewhere (Fisher, '42), that one of the characteristic features of the action of narcotics is the abolition of some cellular functions or activities while others are left relatively intact. Recognition of this generality is of considerable interest for it indicates a special kind of specificity about the action of these inhibitors. Apparently a complex of chemical reactions involving one function of a cell or organism may be stopped while the complex involving some other function, continues. Previous work from this laboratory on yeast (Fisher and Stern, '42), on bacteria (Fisher and Armstrong, '47), (Armstrong and Fisher, '47), on a protozoan (Ormsbee and Fisher, '44) and on sea urchin eggs (Fisher and Henry, '44), (Fisher, Henry and Low, '44) shows that narcotics, such as urethane, in all cases stop growth and arrest part of the oxygen consumption which seems to be associated with it, while leaving most of the oxygen consumption intact. In general it is only the higher concentrations of inhibitor which produce marked inhibitory effects on oxygen consumption and abolish the life of a cell. Such facts, one presumes, provided the basis for the attempts (Paterson, Thomas, Haddow and Watkinson, '46; Kirschbaum and Lu, '46 and Haddow and Sexton, '46) to control tumours and leucaemia with the narcotic urethane.

The existing date, which suggests that narcotics can stop growth at lower concentrations than those necessary to produce marked effects on the overall energy releasing reactions in the cell, deal largely with lower organisms. It was considered desirable to make similar observations on cells of a higher animal. The present study, using Earle's L strain mouse cells (Sanford, Earle and Likely, '48), grown in tissue culture, was therefore undertaken.

MATERIALS AND METHODS

Culture methods. Replicate cultures of Earle's L strain cells were grown in the synthetic media devised by Healy, Fisher and Parker ('54a, '55) supplemented by the addition of 20% horse serum. Both medium number 635 and number 858 (in which, however, the purified coenzymes were replaced by a coenzyme concentrate) were used. There was no indication that the observations recorded here varied with the medium used. The culture techniques employed were those developed by Earle and his associates (Evans, Earle, Sanford, Shannon and Waltz, '51), modified as described by Parker, Healy and Fisher ('54). In general, cultures were carried in Kolle flasks from which the cells were harvested for use in respiration experiments, for the inoculation of cultures in which growth was to be studied and for the inoculation of other Kolle flasks. In the growth experiments approximately 2×10^5 cells contained in 0.1 ml were delivered into "T" flasks and to each was added 2.5 ml of nutrient medium containing, where desired, the appropriate narcotic concentration. Four T flask cultures were prepared at each narcotic concentration and at the end of the growth period three were used for cell counts and one for total nitrogen determination.

When large populations of cells were required to carry out simultaneous analyses of cell volume, total nitrogen, cell count and nucleic acid phosphorus, duplicate cultures were grown in Kolle (K) flasks using medium 858 with 20% horse serum.

In the growth experiments, contrary to the usual practice followed in these methods of tissue culture, no change in nutrient medium was made throughout the week. This reduced the multiplication rate in controls to fourteen fold or less, a rate lower than that attainable when medium is changed. This practice made possible, however, a simple accounting of the disappearance of glucose and the appearance of lactic acid in the nutrient medium.

The number of cells present in the cultures was determined by the counting procedure elaborated by Sandford, Earle, Evans, Waltz and Shannon ('51).

Respiration. The rate of oxygen consumption was measured by Warburg's direct method (Umbreit et al., '51), using 7 ml vessels shaken 80 times per minute through 5 cm. Air was used in the gas phase. On the average 3×10^6 cells were contained in a total volume of 1.6 ml in each vessel. As a suspension medium for the cells Earle's (Earle, Schilling, Straus, Brown and Shelton, '43) inorganic salt solution was used to which 0.08% glucose was added along with 0.02 M phosphate. The pH was adjusted as desired to lie within the range 6.6 to 7.2, within which range the respiration of these cells appeared to be relatively insensitive to changes of pH.

Analytical procedures. Total nitrogen was determined using the micromethod of Snell ('37); glucose was analyzed by the method of Somogyi ('45), lactic acid by the procedure of Umbreit et al. ('51) and desoxyribosenucleic acid phosphorus (DNA-P) by the method of Healy et al. modified for use with L cells ('54b). Cell volume was measured by centrifuging cell suspensions containing known numbers of cells in a thrombocytocrit tube at 2,000 rpm for five minutes.

Morphological examinations. In order to examine live cells under high power phase-contrast microscope, slivers of cover-slip glass were placed in T flasks. Cells climbed on to and divided on the splinters just as if they had been growing on the surface of the culture flask. Splinters were removed daily for examination and prepared on the slide as follows: a sliver placed on a microscope slide was inundated with nutrient medium from the flask from which it had been removed. A cover slip was placed over this and sealed by a wax border to prevent drying.

Cells for electronmicrograph sections were scraped from Kolle flasks, centrifuged, washed and immediately fixed in osmic acid solution. It is a pleasure to record that this material was prepared for electron micrography and the sections were photographed by Dr. A. F. Howatson of the Department of Anatomy, University of Toronto, who used the techniques developed by him for other material (Howatson and Ham, '55).

RESULTS

Effect of luminal on growth and oxygen consumption. Respiration of L strain cells in buffered Earle's solution, with 0.08% glucose added, remains constant for the first hour and 40 minutes and thereafter declines slightly for the remainder of the 3⅓ hour experiment by an average of 10% or less. The average rate of oxygen uptake for the first 1⅔ hours was found in these experiments to be 3.4×10^{-6} mm^3/cell or 2.8 mm^3/μ DNA-P.

In figure 1, in which the relationship between narcotic concentration and respiration is shown, the results are plotted in a manner so as to test the applicability to the data of an expression derived from the mass law. In this method of plotting the respiration in the controls is designated as 100%, U is the uninhibited fraction of the respiration, I the inhibited fraction and U/I the ratio of the two respiratory portions. If the reactions in the cell, of which oxygen uptake is an indicator, obey the mass law and if the narcotic inhibits those reactions in proportion to its concentration then a plot of the logarithm of narcotic concentration against the logarithm of U/I should give a single straight line relationship.

Each value of log U/I corresponds, of course, to a certain per cent inhibition of the oxygen consumption. Therefore, because it was considered more informative, the vertical scale marked in the figure is "percent of normal respiration remaining," rather than "log U/I."

The straight line drawn through the points representing the respiration data in figure 1 can be seen to fit them reasonably well, with the exception of the highest point. It should be pointed out, however, that small errors in the experimental data are magnified by this method of plotting, so that the difference between the highest point and the equivalent value predicted by the line actually represents an experimental error of less than 5%. Within rather small limits, therefore, it can be maintained that the straight line drawn, adequately represents the experimental results.

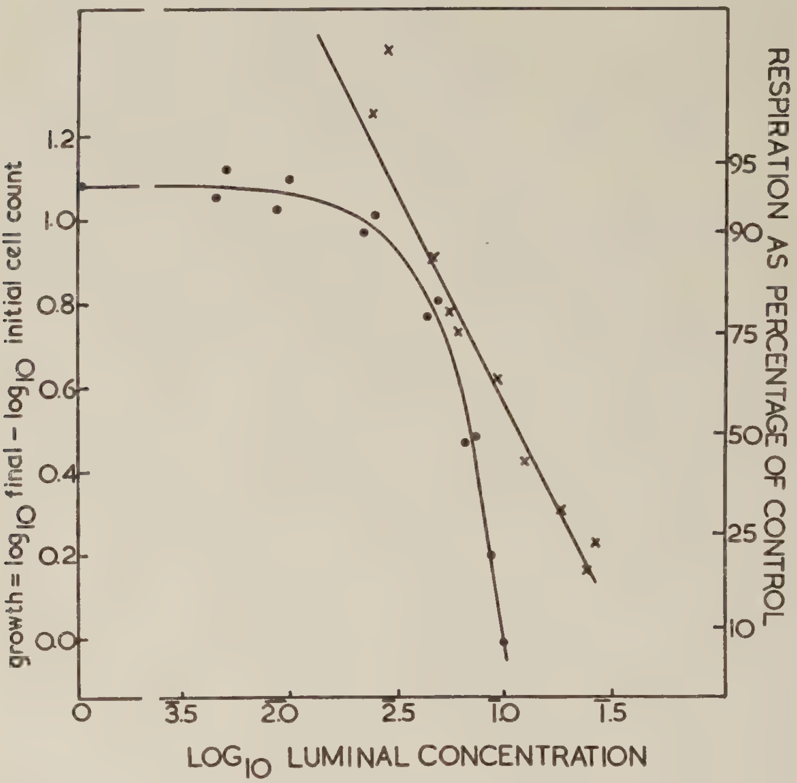

Fig. 1 The rate of growth (dots) and of oxygen consumption (crosses) by L cells at different concentrations of luminal. Details of the experiments will be found in the text.

L cells in control culture multiply fourteen fold or less in a week. The rate of growth will be taken as the difference between the logarithms of the final and the initial cell counts. The effect of luminal on the rate of growth is also shown in figure 1. Each point on the curve is the average of at least

9 individual cultures from three separate experiments. It is evident that growth declines rapidly as the concentration of luminal is increased and that it ceases completely in the presence of 0.1% luminal. In some instances chick embryo extract, one part in 40, was added to the nutrient medium. It was found to increase the rate of cell multiplication slightly at each narcotic concentration but without altering the shape of the growth curve in any way.

It is appropriate at this point to note that the cell counts used throughout this investigation were actually nuclear counts. In the procedure followed in these experiments the cell cytoplasm is dissovled in 2% citric acid, thus releasing the nuclei, which are then counted. It was found in the present investigation and has been reported in at least one other instance (Cailleau et al., '56) that cells treated with inhibitors sometimes become multinucleate. In order to test what portion of luminal treated cells had, in fact, more than one nucleus, direct counts of several thousand cells were made on replicate flasks in which cells had been fixed *in situ*. At the highest and next highest concentration of luminal the percentages of cells found to be multinucleate were 6.3 and 14.5 respectively. The counting procedure adopted here therefore indicated a slightly larger number of cells than was actually present.

At the narcotic concentration at which growth starts to decline the total respiration is inhibited by only 10% but when growth stops completely, 60% of the total respiration still remains. It thus appears that the concentration of luminal which completely prevents cell division does not inhibit very appreciably those metabolic reactions the function of which is manifested by oxygen uptake.

The relatively greater sensitivity of cell division than of oxygen consumption is typically shown in a number of other examples. However the present data are rather unique in that the relationship between narcotic concentration and oxygen uptake, plotted as in figure 1, approximates a single straight line. Thus in yeast (Fisher and Stern, '42), in bacteria

(Fisher and Armstrong, '47; Armstrong and Fisher, '47), in a protozoan (Ormsbee and Fisher, '44), in sea urchin eggs (Fisher and Henry, '44; Fisher, Henry and Low, '44) and in developing frog embryo (Moog, '44) inhibition of oxygen consumption as a function of the inhibitor concentration, when plotted as in figure 1, *cannot* be adequately described by a single straight line. In fact two intersecting lines become a very satisfactory representation of the data. In all these examples, too, cell division is typically reduced to zero at that narcotic-concentration at which intersection of the two lines occurs. The view has been developed elsewhere (Fisher, '42) that these experimental observations can best be explained if one considers that two systems, a resting and an activity one, are functional in the organism. It is the property of narcotics as inhibitors that they abolish the function of the activity system, which can be concerned, for example, with cell growth and mitosis, at concentrations lower than those required to affect the resting system, which may serve to maintain the minimum energy requirements of the cell.

The data for the L cells thus appear at first inconsistent with the findings in these previous instances. It can be shown, however, (Fisher unpublished) that even when two systems are present it is possible, under certain circumstances, to have data closely approximating a single straight line. This can happen in fact whenever the quantitative characteristics of the inhibition of the two systems are similar. In other words the existence of two systems, as suggested in the other examples, remains a possibility, even though the present observations do not reveal it.

Effect of luminal on carbohydrate metabolism. The effect of luminal on glucose disappearance and on the formation of lactate were made in two different situations. First the quantity of glucose disappearing and lactate appearing was determined during culture of L cells in T flasks, following addition of luminal, as described under Methods. The resulting data are shown in the bar diagram of figure 2A. The micrograms of glucose disappearing from each ml of medium are shown by the height of the open rectangles. The black rectangles indicate the quantity of lactic appearing during the culture period, while the height of the stippling indicates the amount of cellular nitrogen present in the culture at the end of the incubation period. Grossly it would appear that as the concentration of luminal was increased the amount of glucose used decreased, as did also the quantity of nitrogen associated with the cells. Both of these observations appear to be consistent with the inhibition of multiplication produced by the narcotic, which was shown above. It is certain, however, that as the luminal concentration was raised lactate production was increased—not inhibited. An acceleration

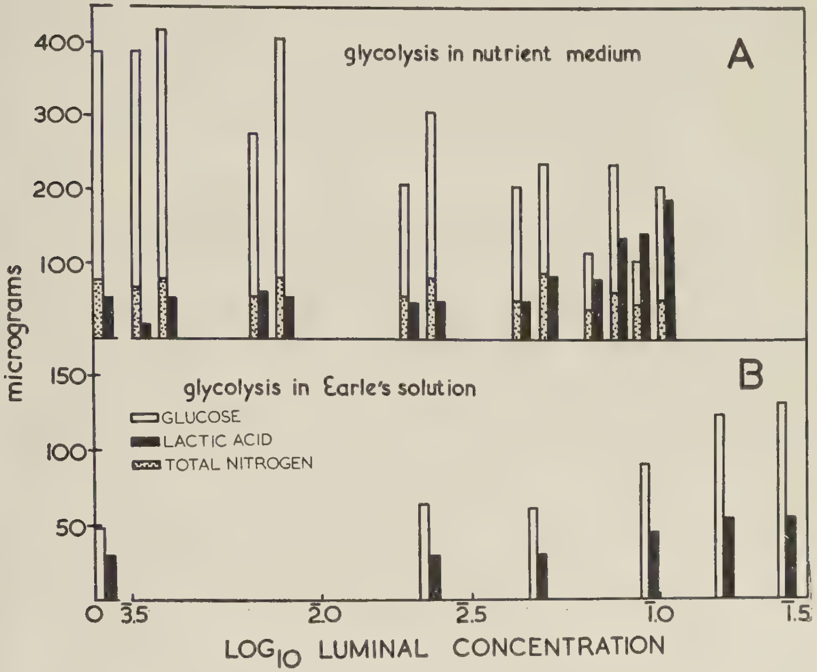

Fig. 2 The effect of luminal on glucose utilization, lactic acid production, and total cellular nitrogen in, (A) growing cultures of L cells and in (B), suspensions of L cells in a salt solution containing glucose. The values in (A) are expressed per 1 ml of culture suspension while the values in (B) are given per 10^6 cells. For convenience in (A) the columns representing the cellular nitrogen have been superimposed upon the columns representing the glucose utilization.

of glucolysis is clearly being caused by the higher concentrations of narcotic. Since the quantity of cellular nitrogen was less at the higher concentrations, the utilization of glucose and the production of lactate, expressed per unit of cellular nitrogen would, of course, be considerably higher than is indicated in the figure. It is appropriate to note also that at the highest luminal concentration the number of cells present was only 1/10 of the number present in the controls. Expressed *per cell,* therefore, the amount of glucose disappearing as well as the amount of lactate appearing was much greater at the highest luminal concentrations than in the absence of the narcotic.

It is of some interest that in the controls the lactate which appears during the culture period accounts for only about 1/6 of the glucose which disappears, but that at the highest concentrations of luminal, nearly all of the glucose which disappears can be accounted for as lactate. In seeking an explanation for this difference it must be recognized that in the control cultures much more oxygen would be used throughout the culture period than in the narcotic-containing flasks. It would be reasonable to assume that, in fact, four times as much oxygen was used in the former instance as in the latter. If we assume that this oxygen is used for burning glucose to carbon dioxide, then the difference between the oxygen used in control flasks and that used when luminal was present should account for the difference in the amount of glucose broken down. Calculations show that it does so to the correct order of magnitude. In order to make the data for the controls and luminal-containing cultures consistent, therefore, it is not necessary to do more than to recognize that as the narcotic concentration is increased glucose is broken down to lactate at an increased rate.

A second set of experiments was carried out to test the effect of luminal on the rate of glucose disappearance when the cells were suspended in Earle's solution in Warburg flasks while the rate of oxygen consumption was being measured. These experiments were of a much shorter duration, lasting only 3⅔ hours, than those made with growing cultures; there was no nitrogen source available in the medium and hence presumably no elaboration of new protoplasm occurred during the course of the experiment. The results given in figure 2B show that the higher concentrations of luminal increase very appreciably the glucose utilization and the production of lactate. At 0.1% luminal, the concentration at which cell multiplication is zero, glucolysis rates are up to about 180% of the control rates. At all concentrations of the narcotic, as well as in the controls, the lactate produced accounts for 40–50% of the glucose disappearing.

It is clear, therefore, that luminal in the range of concentrations which are required to stop cell multiplication, not only fails to inhibit glucolysis but actually accelerates it. If it is true that growth in the L strain proceeds by virtue of the energy derived from glucose catabolism, then the rate of cell multiplication in the presence of luminal was depressed, most probably, because this narcotic interferes with the aerobic oxidative sequence of energy producing reactions.

Effect of luminal on cell volume, total nitrogen and DNA-P. In the foregoing attention has been directed toward cell multiplication as a measure of growth. There are circumstances, however (for example Brace, '50) in which cells form new protoplasm, and hence increase in size, without occurrence of any division, so that there is no increase in cell number. Such observations made it of interest to compare cells from flasks grown in 0.1% luminal with cells grown in the absence of this narcotic, with respect to size, content of DNA-P and total nitrogen. The average data from seven separate experiments, each of which involved two control flasks, with the usual nutrient medium, and two flasks with medium plus 0.1% luminal, are shown in table 1. To assist in judging the significance of the differences between the mean values shown in the table, the probability that each could occur by chance was computed. The probabilities for the difference between the initial values and the controls at the end of the incubation period are given in column (3) of the table, those for the

TABLE 1

Characteristics of L cells in the initial inoculum (column 2), after culture in normal medium (column 4), and after culture in luminal (column 6) [1]

ANALYSIS FOR (1)	INITIAL VALUES (2)	PROBABILITY BY "t" TEST (3)	CONTROLS AT END OF WEEK (4)	PROBABILITY BY "t" TEST (5)	0.1% LUMINAL TREATED AT END OF WEEK (6)
Nuclear count per ml of suspension	259,100 ± 32,900		1,118,000 ± 90,000		334,000 ± 42,300
Cell volume in ml	2.57×10^{-9} ± 0.154	0.50	2.65×10^{-9} * ± 0.118	0.03	3.88×10^{-9} * ± 0.440
μ DNA-P per cell	1.22×10^{-6} ± 0.011	0.13	1.24×10^{-6} ± 0.0020	< 0.01 [2]	1.80×10^{-6} ± 0.420
μ Total nitrogen per cell	7.25×10^{-5} ± 0.79	0.02	4.74×10^{-5} ± 0.29	< 0.01	9.69×10^{-5} ± 1.30

[1] Except for those indicated by an asterisk, which are the average of 6 experiments, each value is the average of results of 7 separate experiments and is accompanied by its standard error.

Each value in column (3) indicates the probability that the values in column (2) and (4), on the same line, could occur by chance alone. Similarly the values in column (5) refer to the differences between the values in columns (4) and (6).

[2] This probability was computed by the analysis of variance to take advantage of the fact that in each of the 7 experiments in this case, duplicate experiments were run. The "t" test, which disregards the measure of reproducibility provided by the duplicates, indicates a probability of only 0.2 in this comparison.

differences between the control and the luminal cultures in column (5). From the first line of the table it can be seen that the nuclear count in the controls increased by more than 330% during the incubation period but by only about 30% in cells treated with luminal. The volume of each cell initially was 2.57×10^{-9} cm^3 and in the controls was 2.65×10^{-9} cm^3, an insignificant change, as was to be expected. After growing in luminal, however, the cell volume was found to be 3.88×10^{-9} cm^3, or 46% larger than in controls—a very appreciable increase and quite significant in the statistical sense.

As was also to be expected, the DNA-P content of the cells initially was the same as that of the cells from the control cultures. After growth in luminal, however, the

DNA-P content became very much more variable, from one culture to another (as indicated by the standard error) and on the average had increased by about 46%, a result which would hardly have been expected considering the constancy which characterizes the DNA-P content of most cells (see, for example, Healy et al., '54b). It is evident, of course, that as a cell commences to grow, its content of DNA-P starts off at some low value and that this content presumably reaches its maximum at the time when the cell divides into two daughter cells. It is probably significant in this connection that the relative increase in cell volume and DNA-P content, occasioned by luminal, parallel one another identically.

A very significant difference also exists between cells cultured in the presence of luminal (column 6) and those grown in control cultures with respect to the total nitrogen content, which more than doubled as a consequence of the presence of luminal. Further evidence of the effect of a variety of concentrations of luminal on the cellular nitrogen was given in figure 2A, which shows that at no concentration did luminal reduce very appreciably the total nitrogen found in all the cells of a culture. At the higher concentrations, however, the narcotic reduced cell multiplication to zero (fig. 1). At these higher concentrations, therefore, the nitrogen content per cell was raised.

It may possibly have been noted already that the cells from control cultures had a lower nitrogen content than did the cells used as the initial inoculum. Such a result appears anomalous and, unfortunately, the full explanation is not known at present. Stock cultures, however, were carried in Roux flasks, while the control and luminal containing cultures were grown in Kolle flasks. From the routines of this investigation there resulted a higher final density of cells (i.e., more cells per ml), other things being equal, in the Kolle flasks than was reached in the Roux flasks. In general it has been our experience that the nitrogen content of L cells from cultures in which a high density of cells is reached, quite definitely tends to be lower than the content of cells from which a lower density of cells is reached. The difference in the amount of nitrogen in the cells of the inoculum and of the control cultures is, therefore, a manifestation of this general experience. A similar observation regarding the nitrogen content of cells from control cultures and cultures containing luminal was also made, for cells from the latter were relatively few in number and contained far more nitrogen than did cells from the controls, in which cell density was much greater.

Effect of luminal on cell morphology. Several instances are now recorded in the literature showing that various inhibitors, including narcotics, produced obvious morphological changes in cells, when the concentration of inhibitor was sufficiently large to abolish most of the cell division (Burt, '45; Bucher, '49; Chèvremont, '53; Cailleau et al., '56). Whether the change in morphology is to be associated with a change in specific chemical reactions concerned with growth, or whether it indicates that some "basal machinery" is being affected, is not clear. In either case it seemed of interest to consider the morphological changes, if any, brought about by treatment with 0.1% luminal. The volume changes which this narcotic produces have already been discussed and were noticeable to us by direct microscopic observation. This may be seen by comparing the phase-contrast micrographs of control cells (fig. 3c) with those of cells from luminal-containing cultures (fig. 3d). It should be noted also that whereas normal cultured cells have smooth and rounded contours, luminal

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Fig. 3 Micrographs of live L cells cultured for one week on splinters of coverslip glass in T flasks with nutrient medium 635 supplemented with twenty per cent horse serum and with added narcotic, when appropriate.

(a) and (b) × 200 approximately; cells from control (a) and 0.1% luminal treated (b) cultures.

(c) × 1,000 approximately; oil phase-contrast; cells from a control culture.

(d) × 550 approximately; high power phase-contrast; cells from 0.1% luminal-treated culture.

(e) × 1,000 approximately; oil phase-contrast; part of a single cell treated with 0.1% luminal to show linear arrangement of mitochondrial particles. Compare with low power micrograph 1 (b).

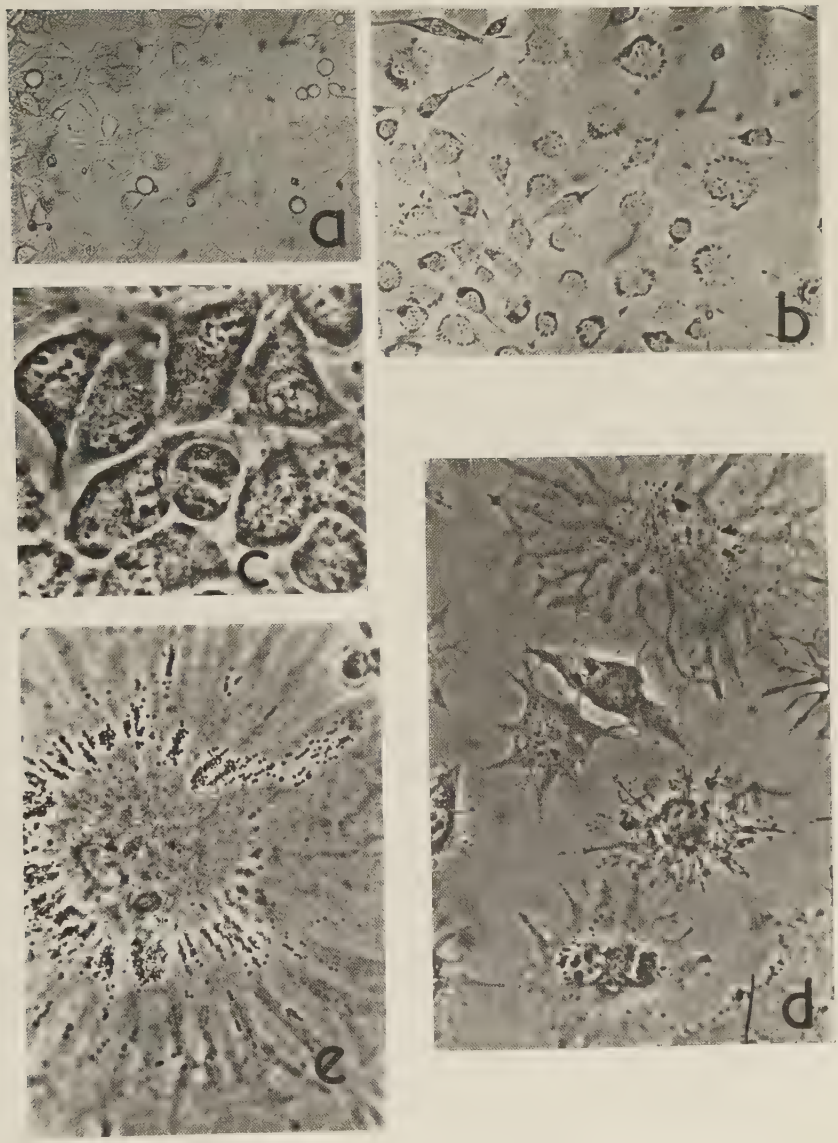

Figure 3

treated ones generally have thick, ridge-like areas of cytoplasm emanating from the center like the spokes of a wheel, with thin, web-like areas of cytoplasm connecting the ridges. Within the cells in figure 3d there are large numbers of spherical inclusions. These seem smaller and much more dense than any similar inclusions in normal cells. The change in cell appearance which these dense particles produce can be seen even more clearly by comparing micrographs (fig. 3, a and b) taken at a lower magnification. From figure 3b one can also conclude that in cells treated with narcotic the particles become arranged regularly, so as to form a circle situated at the periphery of the cell. A high power micrograph of a cell, in which the regular arrangement of the inclusions is particularly well marked, is illustrated in figure 3e. One should note the spherical appearance of the inclusions, the diameter of which ranges from about 0.50 to 0.75 μ. The particles in figure 3e resemble very closely the appearance of inclusions to be found in a large number of micrographs in the current cytological literature. These particles are usually considered to be mitochondria or mitochondrial fragments. From the present work it would seem that cells from luminal-containing cultures had many more mitochondrial fragments than had cells from control cultures. Apparently in the presence of the narcotic many of the mitochondria were broken down. This interpretation is also suggested by an examination of the electronmicrographs of normal and luminal treated cells. The small number, the oval or rounded shape and the regular outline of mitochondria in a normal cell (fig. 4a) is sharply contrasted with the large number of mitochondrial particles of varying sizes and shapes in a narcotic treated cell. Counts made by three independent observers on 10 control and 20 luminal-treated eletronmicrograph cell sections show approximately twice as many particles, with the characteristics of mitochondria, in the treated cells as are present in the controls.

At the magnifications attainable with the phase-contrast microscope it is not possible, of course, to ascertain whether

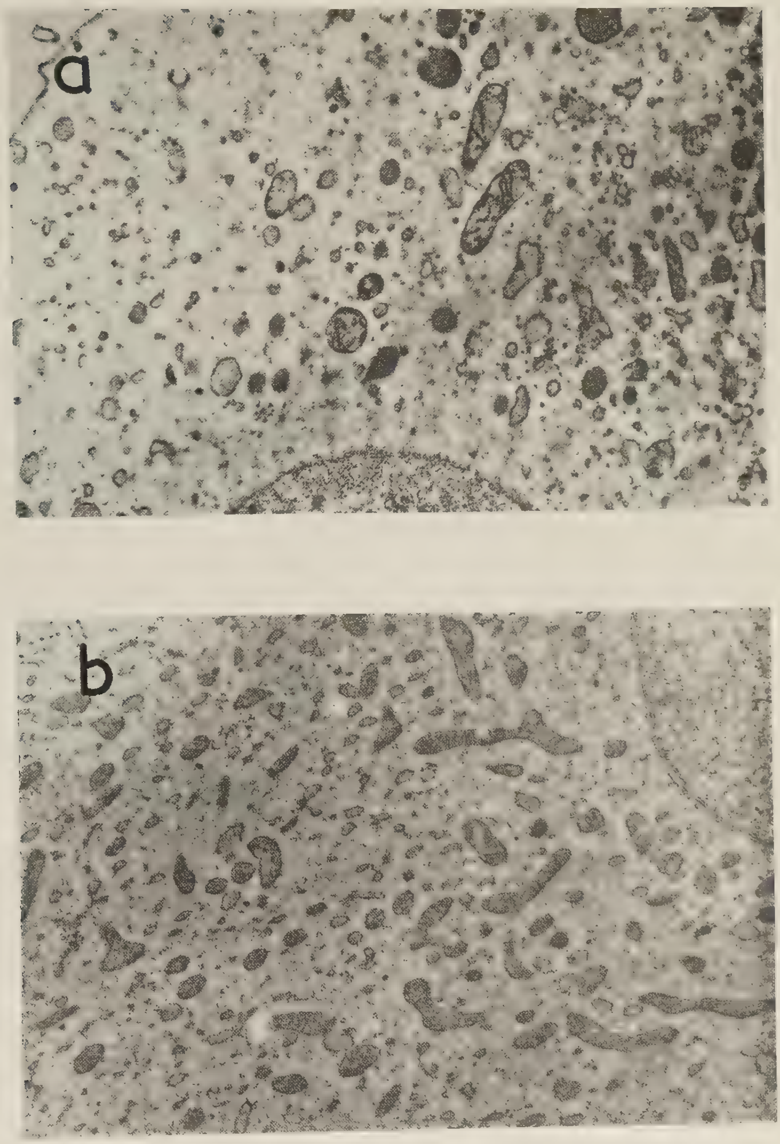

Fig. 4 × 18,000 approximately. Electronmicrographs of sections of the cytoplasm of control (a) L and luminal treated (b) L cells following incubation for five days. Parts of the nuclei are also shown in each case.

the particles distributed in the cytoplasm are, in fact, of mitochondrial origin. A study of the electronmicrographs, however, revealed very few inclusion particles of about the same size as mitochondria but having no internally projecting cristae. These particles, without any obvious internal structure, could be transformed mitochondria. But even if they are particles the nature of which is as yet entirely unknown there were far too few of them to account for the large number of spherical granules seen under high power phase-contrast, in luminal treated cells. In all likelihood, therefore, these regularly arranged, spherical inclusions illustrated in figure 3e are, in fact, mitochondrial particles. It is this change in particle number, concomitant with changes in morphology associated with fractionation of mitochondria, which accounts for the difference between figure 3a and c, micrographs of control cells, on the one hand and figure 3b and d, representing treated cells, on the other. Even in

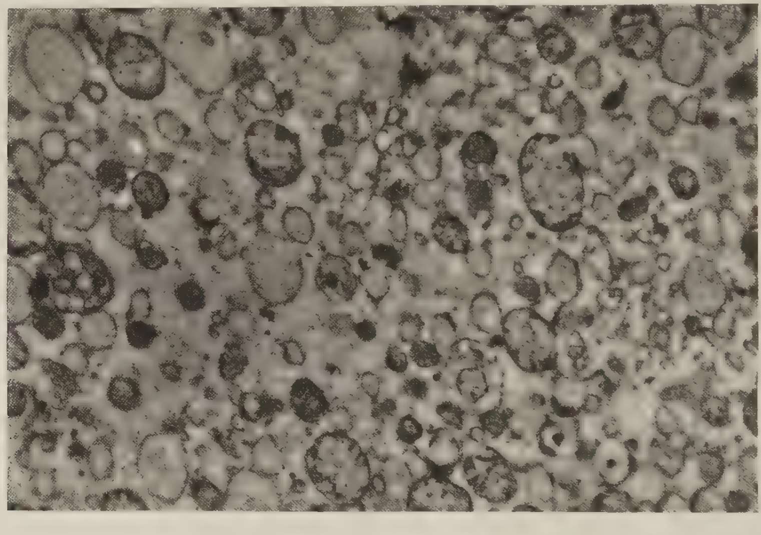

Fig. 5 × 18,000 approximately. Electronmicrograph of a portion of the cytoplasm of a "giant cell" section. Small numbers of these cells are present regularly in control cultures.

isolated mitochondria, subjected to hypotonic conditions, changes from rod-like to spherical forms have been reported by Harman ('50).

Within a normal L strain culture somewhat less than one half of one per cent of the cells are appreciably larger than the rest. These giant cells bear some similarity to the enlarged ones produced by luminal treatment. Figure 5 is an electronmicrograph of a part of such a giant cell. Here too, as happens following luminal treatment, many more mitochondria are present than in cells of normal size. Moreover these mitochondria are vesicular, large, and appear to have abnormal cristae. It is remarkable that an increase in the number of mitochondrial particles was observed both in the "giant" cell and after treatment with the narcotic.

DISCUSSION

A consideration of the literature makes it apparent that morphological changes of the kind described above are not peculiar either to the particular inhibitor employed or to the particular cells used here. Thus, for example, Kirihara ('32) observed that fibroblasts in culture became rounded or polygonal when treated with veronals and luminals. Burt ('45) reported aberrant nuclear behavior during division of a protozoan in the presence of various carbamates and chloral hydrate. Bucher ('49) obtained an increased number of multinucleate and giant cells in connective tissue explants after treating them with urethane. Gilette and Buchsbaum ('55) perfused a steroid through a fibroblast culture and obtained cells with increased numbers of mitochondria (or mitochondrial fragments), and increased cellular and nuclear volumes along with the depression of mitosis. L cells treated with azide are reported by Cailleau et al. ('56) to be enlarged and to contain many cell inclusions, which are referred to as pigmented granules but which are, most probably, mitochondria. From the plates these appear to occur in a regular arrangement similar to that produced by luminal in the present work. Enspherulation of mitochondria in pigeon-breast muscle was produced by Harman and Kitiyakara ('55) by raising the temperature or lowering the external osmotic pressure, as well as by altering the calcium or magnesium concentration of the suspending medium. Dianzani and Scuro ('56) have shown that the long rod-like mitochondria of liver and other tissues can be changed into a spherical form either by direct injection of 2,4-dinitrophenol into the animal or by treating the isolated mitochondria with this substance. X-radiation, too, produces an increase in the size of yeast cells (Brace, '50), in the size of protozoan cells, provided mitosis is stopped (Robertson, '35) and in the size of Ehrlich's ascites tumour cells (Klein and Forssberg, '54). In the last case there is also a concomitant increase in the total nitrogen and pentose nucleic acid of each cell. In fibroblasts, following treatment with urethane, mitochondria become short, grouped and less mobile (Frederic, '54).

It is noteworthy that the various agents and conditions which are thus shown to modify in some way the morphological aspects of mitochondria are also known, in several instances, to interfere with some of the chemical processes which are associated with these cellular particles. Recently Brody and Bain ('54) have shown that barbiturates have an "uncoupling" effect in isolated mitochondrial preparations. Azide (Spiegelman, Kamen and Sussman, '48) and 2,4-dinitrophenol (Lehninger, '55) are also known to uncouple oxidation and phosphorylation. Potter and Bethel ('52) showed that x-radiation of spleen mitochondria reduced their capacity to carry out oxidative phosphorylation. In Harman's experiments ('55) enspherulation of mitochondria was concomitant with an enhanced ATP'ase activity and consequent active dephosphorylation. Dianzani and Scuro ('56) have reported that 2,4-dinitrophenol produces the same effects.

It thus appears that enspherulation, fractionation and immobilization of mitochondria, as well as certain gross changes in cellular morphology, are brought about by the conditions which "uncouple" oxidation from phosphorylation and thus reduce the energy available from substrate degradation. Though mitosis is apparently stopped as a consequence of this decrease in energy availability, the cells may still increase in size and nitrogen content. Perhaps these increases require only a minimal transfer of energy.

The modern conception of the physico-chemical basis for the inhibition by narcotics was provided by the work of Warburg ('21) in which he showed that the activity of his blood-charcoal model of cellular respiration was inhibited by narcotics. Quantitatively these compounds were behaving as though they were being absorbed on the active surfaces of the catalyst, thereby displacing the normal substrates. Following this it began to appear from a variety of studies, of which that by Jowett and Quastel ('37), on brain and other tissues is an example, that it might be the dehydrogenase type of enzyme which was particularly susceptible to narcotics and hypnotics. As factual basis was therefore provided for the proposition that narcotics exerted their effects on intra-cellular catalysts concerned with the liberation of energy. Furthermore the chemical changes concerned in this kind of inhibition involved more closely the immediate substrates consumed, that is the anaerobic end of the oxido-reduction chain, than they did the ultimate reduction of oxygen, that is the aerobic end. Nevertheless it has been shown that some reactions involving atmospheric oxygen could be, in fact, inhibited by narcotics. Thus Keilin and Hartree ('39) using rather high concentrations of urethane point out that this narcotic might form a complex between the dehydrogenase, substrate and cytochrome b (and thereby stop the consumption of oxygen). Michaelis and Quastel ('41) and Grieg ('46) concluded that lower concentrations of narcotic, such as might be effective physiologically, produced a block between the flavoprotein and cytochrome systems. These workers showed, moreover, that this site of narcotic action was more likely to occur *in vivo* than a block of the dehydrogenases, such as that of lactic dehydrogenase for example, the inhibition of which required a much higher concentration of narcotic. It cannot be overlooked, however, that effects of an inhibitor which produce the phenomena of narcosis are effects which can be seen under both aerobic and anaerobic conditions. It was pointed out, as a recent example, that the quantitative characteristics of growth inhibition of yeast by urethane are the same under aerobic and anaerobic conditions (Fisher and Stern, '42) thereby implying that in both circumstances the inhibitor acts on some essential anaerobic chemical reaction. In the observations presented in this paper L cells appear, however, to obtain energy for growth (i.e. cell division) mostly from aerobic chemical changes and when luminal inhibits this growth it presumably does so by inhibiting these aerobic processes.

Much has been learned recently of the importance of high-energy phosphate compounds in connection with the transfer and application of energy in cell function. It is well known that energy can be supplied by both aerobic and anaerobic reactions and that the coupling between energy supplying systems and phosphorylating ones can be destroyed in several ways, as has been noted above. In at least one instance narcotics have been shown to accomplish this uncoupling (Brody and Bain, '54). This kind of effect, in contrast to the inhibitory effects arising from a block in, say, flavoprotein to cytochrome linkage, could conceivably occur under essentially anaerobic conditions. At present, therefore, the "uncoupling" concept merits more ready acceptance as a general explanation of the inhibitory effects of narcotics than does any other. Much more, however, will have to be learned in the future to resolve the general question of how narcotics narcotize and, more particularly, how the "two systems concept," which appears necessary in a logical approach leading to an explanation of narcotic action, is to be accounted for.

SUMMARY

1. The effects of a range of concentrations of the narcotic luminal on growth rate and oxygen consumption of Earle's L strain cells were studied. Growth was calculated from nu-

clear counts and respiration was determined by the conventional Warburg apparatus.

2. In the presence of 0.1% luminal growth ceased completely, although 60% of the total respiration remained uninhibited. The quantitative relationship between the concentration of luminal and its effect on oxygen consumption was adequately accounted for, on the basis of the mass law, by assuming a single site for the action of luminal. In most other cases which have been studied two sites must be assumed to account for the observed relationship between concentration and effect.

3. Both during growth in nutrient medium and during respiration in buffered salt solution, with added glucose, luminal increases the quantity of glucose disappearing and lactic acid accumulating. This suggests that the narcotic interferes with energy-generating mechanisms within the cell which normally control glucose breakdown. Several of the actions of narcotics in cells were discussed. Of these the uncoupling of high-energy phosphate generating reactions from the energy supplying ones has the characteristics which appear to account, in a general way, for the site of action of narcotics.

4. Incubation of L cells in the presence of 0.1% luminal for one week produced a variety of changes in them. In general appearance they differed markedly from control cells. Measurements showed an increase in the volume of each cell and an increase in total nitrogen per cell. An average of 6.3% of the treated cells became multinucleate. Analyses for DNA-P showed an increase per cell due to the presence of luminal.

5. Electromicrograph studies of the cytoplasm of treated cells revealed a break-up of mitochondria into many particles of varying size and shape, whilst phase-contrast microscopy indicated a regular arrangement of spherical inclusions, considered most probably to be of mitochondrial origin. These changes in cell morphology are discussed in relation to changes in energy producing systems.

ACKNOWLEDGMENTS

This research would not have been possible without the advice and active cooperation given freely by Dr. Raymond C. Parker and various members of his laboratory, especially Mr. George Healey. It is a pleasure to acknowledge the contribution of that group. We wish also to express our appreciation of the manner with which our assistant, Mrs. Mary Truesdale, discharged her responsibilities. Finally we wish to record the important role in the success of the investigation played by the grants-in-aid to Kenneth C. Fisher from the Ontario Cancer Treatment and Research Foundation, the Foster Bequest and the Banting Foundation.

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BIOCHEMICAL AND PHYSIOLOGICAL DIFFERENTIATION DURING MORPHOGENESIS

XXII. OBSERVATIONS ON AMINO ACID AND PROTEIN SYNTHESIS IN THE CEREBRAL CORTEX AND LIVER OF THE NEWBORN MOUSE [1]

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FIVE FIGURES

The observations reported here were made on the living, newborn mouse. An effort was made to gain estimates of the following quantities in cerebral cortex and liver: (a) the rate at which the carbon of tissue glutamic acid, a non-essential amino acid, is derived from blood glutamic acid; (b) the rate at which the carbon of several non-essential amino acids of the tissues is derived from blood glucose; (c) the rate at which the carbon of certain essential amino acids of the tissues is derived from these amino acids in the blood; and (d) the rate at which the pools of these amino acids in the tissues are drawn upon for protein synthesis. This is the first step in a study of the effect of growth and maturation on these quantities.

METHODS

Glucose uniformly labeled with C^{14} (glucose-U-C^{14}) was prepared from $BaC^{14}O_3$, $C^{14}O_2$ being synthesized into sugars by *Canna* leaves (Udenfriend and Gibbs, '49; Putnam and

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[1] This investigation was aided by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[2] We are grateful to Miss Sophie Wysienska for technical assistance.

Hassid, '52). The glucose was repeatedly chromatographed in phenol/water/ammonia (80:20:0.3) until radioactive contaminants (largely fructose) were reduced to less than 1%; phenol was removed from the area of the chromatogram occupied by glucose by running the paper in sec.-butyl alcohol/formic acid/water (70:10:20). With our counting arrangement, using a thin window Geiger tube, the glucose had a specific radioactivity of 3.8×10^4 counts/minute/microgram carbon. Randomly labeled l-amino acids were obtained by growing *Chlorella pyrenoidosa* with $C^{14}O_2$ ($+ CO_2$) as sole carbon source (Roberts, Cowie, Abelson, Bolton and Britten, '55); after a 10-fold increase in cell mass the protein was separated, hydrolyzed and two-dimensional ascending chromatograms run first in sec.-butyl alcohol/formic acid/water followed by phenol/water/ammonia. After locating the amino acid spots with radioautograms, the spots were cut out, phenol removed by repeated extraction of the paper with equal parts of acetone and ether, the amino acids eluted and then tested for purity by two-dimensional chromatography in the solvents previously used. The amino acids had a specific radioactivity of 2.5×10^4 counts/minute/microgram carbon.

Newborn mice weighing 1.3 to 1.5 gm were injected subcutaneously over the flank with a glass micropipet containing 5.0 microliters of the radioactive material. In experiments lasting longer than two hours animals were returned to the mother shortly after the injection. Adequacy of feeding was checked by observing milk in the stomach through the relatively transparent abdominal wall. At the end of the experiments the animals were placed on ice until spontaneous movements ceased. The chest was then opened, the heart incised and about 60 mg of blood were collected from the thorax in a small amount of heparin. The blood samples were then mixed with 4 ml of ice cold 5% trichloroacetic acid (TCA). Cerebral cortex and liver were promptly removed and 50 mg of each immediately homogenized in 4 ml of 5% ice cold TCA. The subsequent procedure has been fully described (Roberts, Cowie, Abelson, Bolton and Britten, '55). In brief, after 30 minutes at 5° the homogenate was centrifuged and the supernatant fluid poured off. This is the cold TCA-soluble fraction and is assumed to contain the pool amino acids of the tissues. The precipitate was stirred in 4 ml of 75% ethanol, kept at 40–50° for 30 minutes and then centrifuged. The supernatant fluid was poured off, giving the alcohol-soluble fraction. The precipitate was stirred in 4 ml of solution containing equal parts of ether and 75% ethanol. After 15 minutes at 40–50° the suspension was centrifuged. The supernatant fluid is the alcohol-ether-soluble fraction and was combined with the alcohol-soluble fraction. The precipitate was stirred in 4 ml of 5% TCA and kept in a boiling water bath for 30 minutes and centrifuged. The supernatant fluid is the hot TCA-soluble fraction. The remaining precipitate was washed free of residual TCA, stirred in acidified ethanol and centrifuged; the precipitate was then stirred in ether and centrifuged. The supernatants from these two washes were discarded. The precipitate is the TCA-insoluble or protein fraction. It was demonstrated to be free of glutathione.

In this report we shall confine ourselves to the cold TCA-soluble fraction and the TCA-insoluble fraction. The TCA from the cold TCA-soluble fraction was removed by shaking 5 times each in two volumes of ether and the ether discarded. This material was further fractionated using the cation exchange resin, Dowex 50-X8, in the acid form. In experiments with radioglucose, the fraction containing glucose and lactate which passed through the column was collected and subsequently chromatographed in sec.-butyl alcohol/formic acid/water. Amino acids were eluted from the column with 6 column volumes of 4 N NH_4OH and the ammonia from this fraction driven off on a hot plate. Protein precipitates from cortex and liver were hydrolyzed in 0.3 ml 6NHCl at 106° for 16 hours. Aliquots of the protein hydrolysate corresponding to 20 mg cortex and 10–15 mg liver as well as the total amino acid portion of the cold TCA-soluble fraction were then each dried in a current of air and subsequently taken up in a drop of H_2O_2 for transfer to the chromatogram paper. Unlabeled protein hydrolysate from liver or cortex and glutamine were added to the chromatograms of the amino acids of the cold TCA-soluble fraction for subsequent ease of identification of amino acids when treated with ninhydrin; γ-amino butyric acid was also added to the fraction from cortex. The fractions were then run on two-dimensional, ascending chromatograms; first in sec.-butyl alcohol/formic acid/water followed by phenol/water/ammonia. Radioautograms were made of the finished chromatograms and the radioactivity of the indicated spots counted directly from the paper. The chromatograms were then treated with ninhydrin for final identification of the spots.

Chromatograms were made of the amino acid portion of the cold TCA-soluble fraction of blood, cortex and liver and of the hydrolyzed protein of cortex and liver. In the experiment with labeled glucose, chromatograms were also made of the glucose-lactate portion of the cold TCA-soluble fraction from blood, cortex and liver. The usual procedure was to count aliquots of fractions in cups and then to determine the proportionate radioactivity of components by counting the spots on the chromatograms; in some instances shortage of material permitted only one of these two procedures. Cup values only are plotted in the graphs to permit comparison among experiments.

Whole blood has been used rather than plasma because of shortage of material and because we have found that for our purposes there is not an important difference in the distribution between plasma and erythrocytes of the tagged amino acids we have used. Distribution ratios have been measured in blood taken from animals after injection of the tracer as well as in samples of blood to which tracer was added *in vitro* (Christensen, Riggs and Ray, '52). Plasma concentrations on occasion were 15% higher than those of cells; in other instances, the two were indistinguishable.

As has been stated, samples of liver and cortex were obtained after bleeding the animal from the heart. The contamination of labeled tissue protein by labeled blood protein does not appear to have been a significant source of error. With labeled glutamic acid the protein of 100 mg blood never contained more than a fraction of a per cent of the radioactivity of the same weight of tissue; with labeled essential amino acids, the protein from 100 mg blood varied from 15% (15 minute sample) to a maximum of 25% (one hour sample) of the radioactivity of 100 mg cortex and from 3% (30 minute sample) to 15% (15 minute sample) of the radioactivity of 100 mg liver. Inspection of the samples of cortex indicated that they contained but little blood.

RESULTS

Injection of l-glutamic acid-U-C^{14}. This series consisted of 4 littermates which were sacrificed 10, 30, 60 and 240 minutes after subcutaneous injection of uniformly labeled glutamic acid. The first three animals received 1.2×10^5 counts/minute; the last, 10 times this amount. Observations are given in table 1 and figure 1.

One of the aims of these experiments was to estimate the rate at which carbon of blood glutamic acid appears in glutamic acid of cortex and liver. If the blood glutamic acid is an immediate precursor of the glutamic acid of the tissues, that transferred from blood to tissue will have the same specific radioactivity as that in blood. If an intermediate pool such as blood glutamine is present, however, it will dilute the radioactivity of carbon derived from blood glutamic acid and calculations of rates of transfer to tissue based on the assumption that blood glutamic acid is an immediate precursor may be less than the actual rate. Evidence that intermediate pools do not seriously affect rates of transfer calculated on this assumption is provided by finding substantially equal rates at successive periods of time. In such a case, intermediate pools either do not exist to an important degree or their specific radioactivity quickly reaches that of the precursor substance. On this basis and because of the finding that the radioactivity of blood glutamine rises slowly and is

TABLE 1

Analysis of several chromatograms from experiments with l-glutamic acid (designated G) and with l-phenylalanine, l-leucine and l-isoleucine (designated P)

Cold TCA (amino acids of pools) refers to amino acid fraction eluted from Dowex-50 with ammonia. % = percentage of radioactivity of all amino acids of sample present in indicated amino acid. S = shadow, radioactivity too low for counting; ph. alanine + = phenylalanine + leucine + isoleucine; tyrosine + = tyrosine + tyrosine oxide.

AMINO ACID	BLOOD (G) COLD TCA		LIVER (G) COLD TCA		CORTEX (G) COLD TCA		LIVER (G) PROTEIN	CORTEX (G) PROTEIN	BLOOD (P) COLD TCA	LIVER (P) COLD TCA	CORTEX (P) COLD TCA	LIVER (P) PROTEIN	CORTEX (P) PROTEIN
	1 hr. %	4 hr. %	1 hr. %	4 hr. %	1 hr. %	4 hr. %	4 hr. %	4 hr. %	4 hr. %	4 hr. %	4 hr. %	4 hr. %	4 hr. %
Alanine	S	S	S	1		1	8	13		S		S	2
Arginine	S	S	S	S	S	S	14	3					
Aspartic ac.	S	S	9	3	S	17	11	15		S	S	1	2
Cysteic ac.			S	6		S							
γ-am. butyric ac.					S	6					S		
Glutamic ac.	83	53	60	57	58	52	58	62		2	17	2	3
Glutamine	17	43	31	25	42	21				S	S		
Glycine				S		S	3	S					
Lysine		S											
Ph. alanine +	S				S				100	98	83	92	93
Proline	S	4	S	3	S	3	4	5					
Serine				3		S	1	3					
Threonine							S	S					
Tyrosine +												5	

only 17% of the radioactivity in the blood at the end of one hour (table 1), we have considered blood glutamic acid to be an immediate precursor of the glutamic acid of the tissues.

Taking blood glutamic acid carbon as equal to 1.2 μg/100 mg blood (Albritton, '52), its specific radioactivity was obtained by using the average value of the blood radioactivity-time curve of figure 1 for the desired time interval. Tissue glutamic acid is not a truly stable end product but is degraded and converted to a number of other compounds. At one hour, however, the only known important product is glutamine (table 1). Accordingly we have taken the sum of the radioactivity recovered in glutamic acid plus glutamine of pool and glutamic acid of protein as representing the end products of blood glutamic acid in the tissues.

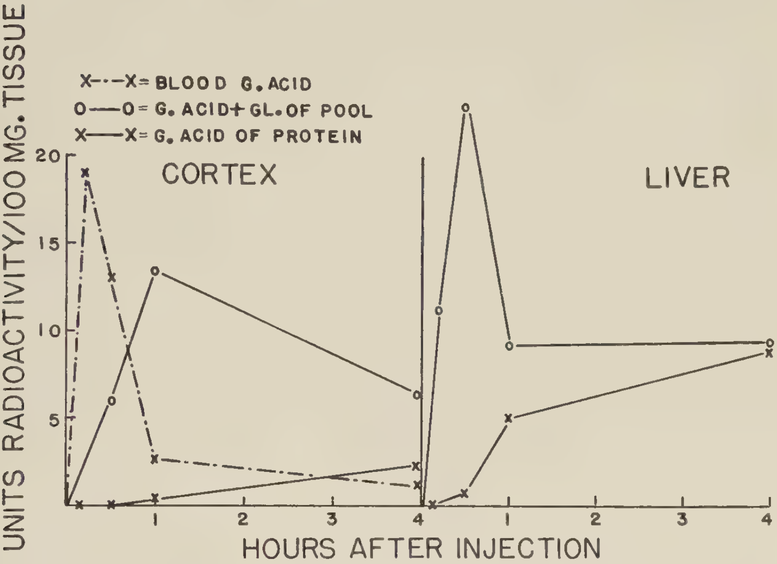

Fig. 1 Radioactivity of glutamic acid (G. Acid) in blood and in protein of cortex and liver and of glutamic acid plus glutamine (Gl) in amino acid pool of cortex and liver, following subcutaneous injection of l-glutamic acid-U-C^{14}. One unit of radioactivity = 64 counts/minute. Since the quantity of tagged glutamic acid in the animal sacrificed at 4 hours was 10 times that used in others of the series, measured values at 4 hours have been correspondingly reduced for this graph.

Using the values of figure 1 at 10 and 30 minutes for the liver and at 30 and 60 minutes for cortex, the following rates were obtained for the transfer of glutamic acid carbon from blood to glutamic acid plus glutamine of liver and cortex, all in microgram C/100 mg tissue/hour: for the liver, 6.0 and 4.2, respectively; for the cortex, 1.1 and 1.6.

A second aim of the experiments was to estimate the rate at which the pool of glutamic acid plus glutamine furnishes amino acid to protein. Since any glutamine which may be present in protein is isolated after acid hydrolysis as glutamic acid, only the sum of the two amino acids in intact protein is known. As an approximation we have consequently considered the pool of glutamic acid plus glutamine as the precursor of glutamic acid recovered from protein. The glutamic acid and glutamine newly incorporated into protein during a given time interval will have the same specific radioactivity as the material of the pool. Accordingly, the rate of incorporation into protein can be expressed in terms of pool size by comparing, during an interval of time, the increment of radioactivity in protein with the average radioactivity of the pool.

Using the increment in tagged carbon present in protein between 30 and 60 minutes (fig. 1), the following values were obtained for the rate at which the combined pool (p) of glutamic acid and glutamine is drawn upon for protein synthesis: for the liver, 0.6 p/hour; for the cortex, 0.1 p/hour, i.e., in the liver 60% and in the cortex 10% of the combined pool of glutamic acid and glutamine appears to be incorporated in protein per hour.

Injection of glucose-U-C^{14}. This series consisted of 5 littermates which were sacrificed at 17 minutes, 1, 2, 4 and 7 hours after subcutaneous injection of 2.3×10^4 counts/minute of glucose-U-C^{14}.

The radioactivity of glucose and lactate in the glucose-lactate portion of the cold TCA-soluble fraction was measured after chromatography. Of the total radioactivity in glucose and lactate, glucose contained the following percentages: for blood, 97–99% except for 88% at two hours; for cortex, 53–56% except for 78% at 7 hours; for liver 90–95% except for 83% at two hours.

With the same approach used for the analysis of observations on glutamic acid, the total amount of tissue amino acid carbon derived from blood glucose/unit time/unit weight of liver or cortex was estimated from the observations of figure 2. Using the values at 17 minutes and one hour (the radioactivity of the amino acids of the pool was added to that of protein) and the value of 100 mg glucose/100 ml blood, which is the average we have found for the newborn mouse, the following rates were obtained for incorporation of blood glucose carbon into carbon of tissue amino acids, all in microgram carbon/100 mg tissue/hour: for the liver, 13 and 14, respectively; for the cerebral cortex, 11 and 19.

We have also been interested in comparing the amount of carbon of an individual amino acid derived by liver or cortex from the amino acid in the blood with the amount of the amino acid synthesized *in situ* from glucose; in this we are presently limited to glutamic acid. It is evident from figure 1 and from the blood level of glutamic acid and glutamine given in figure 2 that the amount of C^{14} derived by either cortex or liver from labeled glutamic acid in the blood in the experiment with labeled glucose is inconsequential. On the basis of the one-hour measurements of figures 3 and 4, the following rates in terms of microgram carbon/100 mg tissue/hour were obtained for incorporation of glucose carbon into carbon of glutamic acid plus glutamine: for the liver, 6.4; for the cerebral cortex, 13. It consequently appears that glucose supplies 10 times more carbon than does glutamic acid of blood to glutamic acid and glutamine of the cortex and an equal amount in the liver.

Finally, again with the same approach used for glutamic acid, the measurements of figures 3 and 4 permit an estimate of the rate at which the individual amino acid pools (p) are drawn upon for protein synthesis:

AMINO ACID	LIVER/HR.	CORTEX/HR.
Glutamic acid + glutamine	0.8 p	0.1–0.2 p
Alanine	1.5 p	2.2 p
Aspartic acid	4.2 p	0.3 p
Glycine	2.4 p	
Serine	2.8 p	0.7 p

These calculations for the liver have been made from the increments in radioactivity of amino acids of pool and protein between 17 minutes and one hour; subsequent observations yield a substantially smaller value for the apparent rate at which the pools provide amino acids for protein synthesis. By contrast, in the cortex there is good agreement among rates calculated from observations on animals at 1, 2 and 4 hours, e.g., these rates for aspartic acid are 0.3, 0.2 and 0.3 p. It will be noted that this experiment with labeled glucose and that with labeled glutamic acid give substantially the same rates for incorporation of glutamic acid plus glutamine of pool into protein of liver and cortex.

Injection of l-phenylalanine-U-C^{14}, l-leucine-U-C^{14} and l-isoleucine-U-C^{14}. These three amino acids are not completely separated from one another by the solvents which we have used for chromatography and have consequently been used as a group to study the behavior of essential amino acids. Since the ratio of carbon of phenylalanine to that of leucine and isoleucine is practically the same in the blood of the mouse (Albritton, '52) as in hydrolysate of *Chlorella* protein (Roberts, Cowie, Abelson, Bolton and Britten, '55), it appears that their specific radioactivity in the blood, at least immediately after injection of the labeled amino acids, would be substantially equivalent.

The series consisted of 4 littermates sacrificed at 15, 30, 60 and 240 minutes after subcutaneous injection of the uniformly labeled amino acids. The first three animals received 1.1×10^5 counts/minute; the last, 9.4 times this amount. The observations are given in figure 5 and table 1.

Comparison of figures 1 and 5 show several differences between the behavior of glutamic acid and the three essential amino acids (the figures have been plotted on the basis of equal initial amounts of radioactivity per unit weight animal

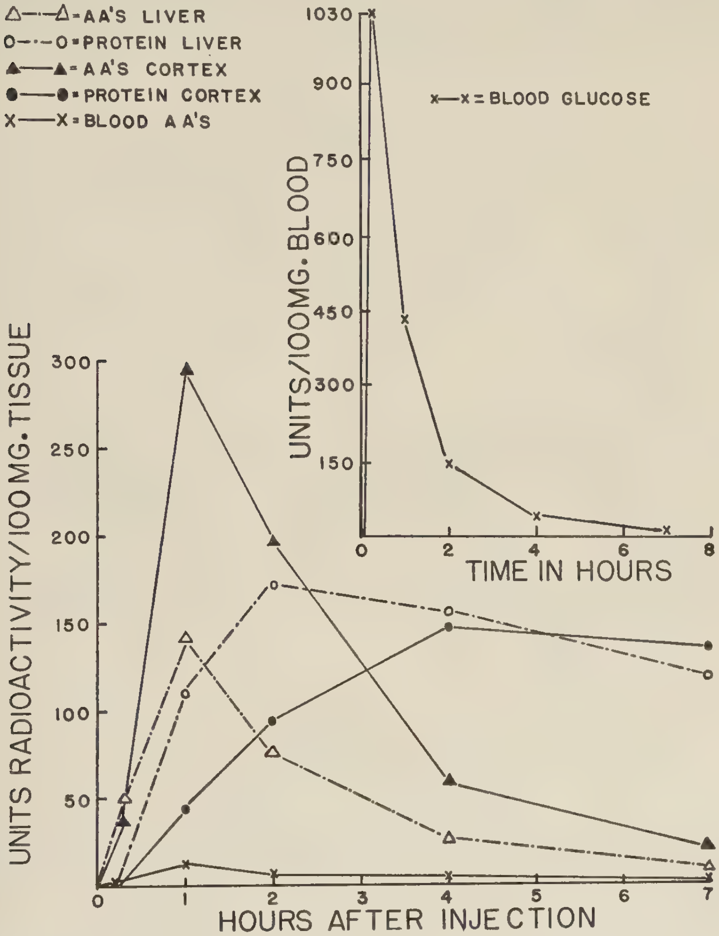

Fig. 2 Radioactivity of glucose (insert) and total amino acids/100 mg blood and of the total amino acids of pool and hydrolyzed protein/100 mg liver or cerebral cortex following subcutaneous injection of glucose-U-C^{14}. One unit of radioactivity = 64 counts/minute. A chromatogram of the blood at two hours showed the following amino acids in the indicated percentage of the total radioactivity: aspartic (shadow too low for counting), glutamic acid (18%), serine (25%), glycine (18%), alanine (37%), proline and glutamine (both shadows).

and so are immediately comparable). The blood level of tagged essential amino acids fell more rapidly from its peak than did glutamic acid. Correspondingly, the total tagged amino acid in the tissue (pool + protein) rose more rapidly and to higher levels with the essential amino acids than with glutamic acid. Incorporation of tagged essential amino acids into pro-

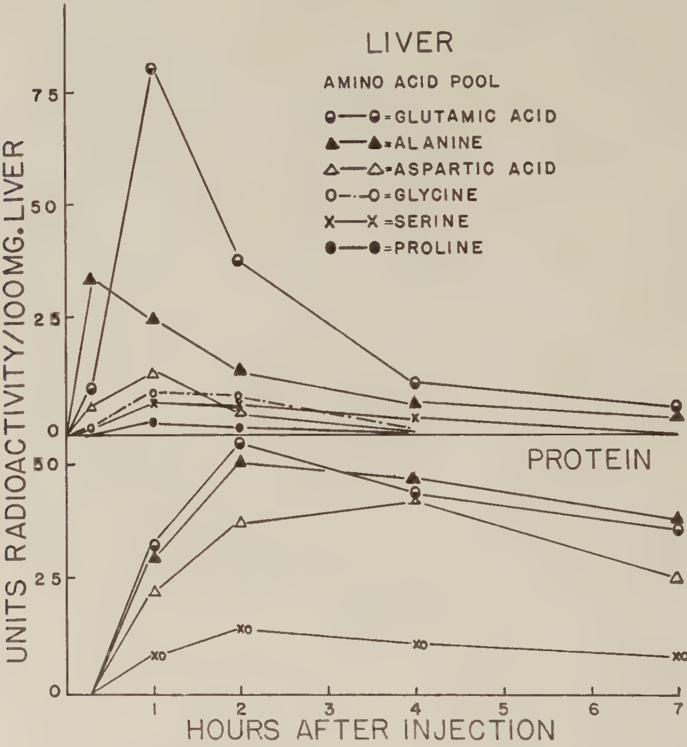

Fig. 3 Radioactivity of amino acids of amino acid pool and of protein hydrolysate of liver following injection of glucose-U-C^{14}. One unit radioactivity = 64 counts/minute. In addition to the amino acids of the figure the following were found to contain C^{14} in the cold TCA-soluble fraction: cysteic acid (shadow at 1 hour; at 2 hours, 1.4; 4 hours, 0.8; 7 hours, 0.8 unit radioactivity); proline (at 1 hour, 0.7; 2 hours, 0.5; 4 hours, 0.2 unit radioactivity and shadow at 7 hours); glutamine (shadow at 17 minutes, 4 and 7 hours; at 1 hour, 0.7; 2 hours, 0.5 unit radioactivity); aspartic acid and glycine (shadows at 4 hours); aspartic acid and serine (shadows at 7 hours). The protein hydrolysate had proline (shadows at 1, 2 and 4 hours; at 7 hours, 0.5 unit radioactivity); arginine (shadows at 1, 2 and 4 hours; at 7 hours, 1 unit radioactivity); cysteic acid (shadows at 4 and 7 hours). Shadows were spots too low for counting.

tein was at a greater rate and reached higher levels than with glutamic acid. No tagged glutamic acid was demonstrable in protein of cortex at 30 minutes and none in protein of liver at 10 minutes; by contrast, there was a substantial amount of

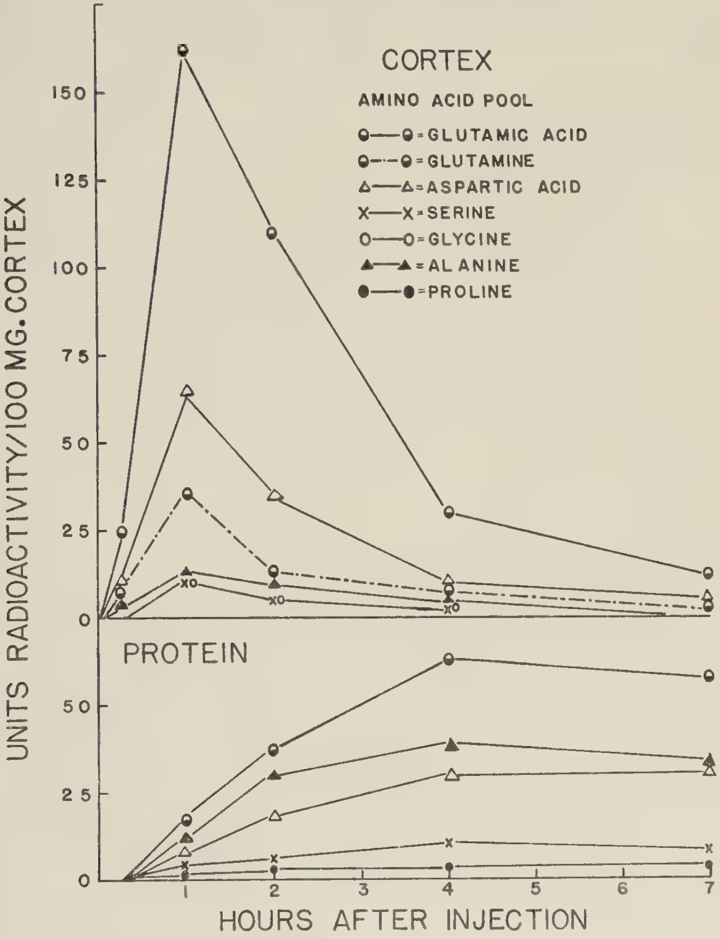

Fig. 4 Radioactivity of amino acids of amino acid pool and protein hydrolysate of cortex following injection of glucose-U-C^{14}. One unit radioactivity = 64 counts/minute. In addition to the amino acids of the figure, the following were found to contain C^{14} in the cold TCA-soluble fraction: proline (shadows at 1 and 4 hours; at 2 hours, 1 unit radioactivity); γ-amino butyric acid (shadow at 1 hour; at 2 hours, 6; at 4 hours, 4; at 7 hours, 1 unit radioactivity). The protein hydrolysate also contained glycine (shadows at 1 and 2 hours; at 4 and 7 hours, 1 unit radioactivity) and histidine (shadows at 4 and 7 hours).

tagged essential amino acids in protein of both tissues at 15 minutes. This difference with respect to rate of incorporation into protein was reflected in the behavior of the amino acid pools. In the experiment with tagged glutamic acid, that present in the pool of the cortex was considerably greater throughout 4 hours than that in protein; the same held true for liver, at least up to one hour. By contrast, after 30 minutes in the cortex and 15 minutes in the liver, the greater part of the tagged essential amino acids was in protein.

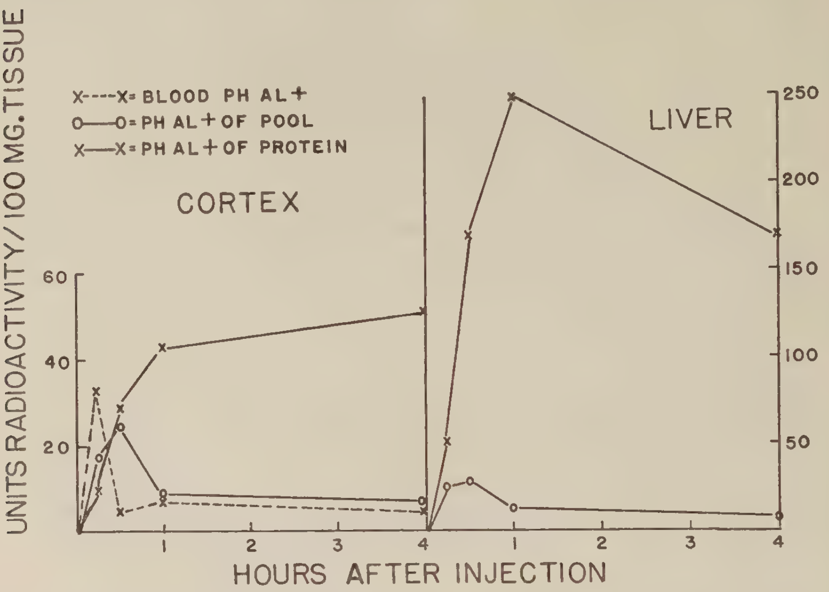

Fig. 5 Radioactivity of phenylalanine, leucine and isoleucine in blood and in amino acid pool and protein of cortex and liver following subcutaneous injection of these amino acids uniformly labeled with C^{14}. One unit of radioactivity = 64 counts/minute. The ordinate on the left is for cortex and blood; that on the right, for liver. Ph. Al. + = phenylalanine + leucine + isoleucine. Measurements at 4 hours have been adjusted for the larger quantity of tagged amino acids injected at this time. To permit direct comparison with the results obtained with glutamic acid (fig. 1), all measurements have been adjusted to the same quantity of radioactivity per unit weight of animal used with glutamic acid.

The difference in behavior of the essential amino acids and glutamic acid is further shown by calculations of the kind used above for glutamic acid. Per unit volume of blood, the total carbon of the three essential amino acids is three times that of glutamic acid (Albritton, '52). To compare rates of transfer of blood amino acid carbon to tissue carbon it is consequently necessary to refer all calculations to a unit weight of carbon of blood amino acid. The results (table 2) show that carbon of the essential amino acids appears 3 to 8 times as rapidly in these amino acids of cortex and liver and 50 to 80 times as rapidly in protein as does that of glutamic acid of blood.

TABLE 2

Rate of transfer of blood carbon in terms of micrograms carbon per 100 mg tissue per hour per microgram carbon of amino acid of blood to total amino acids (pool + protein) and to protein of liver and cortex

The indicated times refer to times after injection of the tracer.
Ph. alanine + = phenylalanine + leucine + isoleucine

AMINO ACID	LIVER TOTAL AA'S		LIVER PROTEIN		CORTEX TOTAL AA'S		CORTEX PROTEIN	
	30′ μgm C	60′ μgm C	30′ μgm C	60′ μgm C	30′ μgm C	60′ μgm C	30′ μgm C	60′ μgm C
Glutamic acid (1)	3.5			0.5	0.9	1.3		0.05
Ph. alanine + (2)	26	24	23	23	7.0	4.3	4.0	3.8
(2) ÷ (1)	7.5			46	7.8	3.3		76

The high rate of incorporation of the tagged essential amino acids into protein is reflected in the rate at which the amino acid pool is drawn upon for protein synthesis. Using the observations at 15, 30 and 60 minutes, the rate for the liver per hour is 16 p, 17 p and 15 p, respectively; for the cortex, 4.7 p, 4.5 p and 3.6 p, i.e., in the liver an amount of amino acid equal to that present in the pool is incorporated into protein in about 6 minutes; in the cortex in about 15 minutes.

DISCUSSION

The liver and cerebral cortex of the newborn mouse utilize the carbon of glucose for substantial synthesis of non-essential amino acids. A complete, quantitative analysis of the contribution made by all sources to any one of these amino acids is lacking but the importance of glucose is made evident by the finding that it supplies 10 times more carbon than does blood glutamic acid to glutamic acid and glutamine of the cortex and an equal amount in the liver. Comparable observations with labeled glutamine and other amino acids are necessary to complete the picture. The finding that glutamic acid carbon is derived from phenylalanine, leucine and isoleucine (table 1) indicates its widespread origin.

The difference in rate of incorporation into protein of the tagged essential amino acids and tagged glutamic acid depends in part upon the flow of glucose carbon into glutamic acid and glutamine, and in part upon the difference in concentration of the amino acids in their respective pools. Tagged glutamic acid entering the tissue from the blood is diluted by untagged glutamic acid synthesized *in situ* from glucose. Additional dilution of tagged glutamic acid is caused by the high concentration of this amino acid in the amino acid pool. It has been found in the brain of the adult rat that the molar concentration of glutamic acid is 130X and glutamic acid plus glutamine 200X that of phenylalanine, leucine or isoleucine (Weil-Malherbe, '52). While we have not made quantitative measurements of the pools in the newborn, ninhydrin color values show clearly that glutamic acid and glutamine in both liver and cortex are in very high concentration and the three essential amino acids, in low concentration. Tagged glutamic acid for these two reasons is incorporated slowly into protein and the tagged essential amino acids, rapidly.

The most striking difference in these experiments between the behavior of liver and cortex appears to be in the rate at which the amino acid pools are drawn upon for protein synthesis. With the exception of alanine, all the pools of the liver which we have studied furnish a higher proportion of their amino acid per unit time to protein than in cortex. The greatest difference is with aspartic acid where the turnover rate in the liver is 14 times that in the cortex; with glutamic acid, serine and the essential amino acids, the rate in the liver exceeds that in the cortex by a factor of about 4. Whether this means a greater rate of incorporation of these amino acids into hepatic protein must wait upon determination of concentrations of amino acids in the pools of cortex and liver of the newborn animal.

Winzler, Moldave, Rafelson and Pearson ('52) have reported on the tagged amino acids found in protein hydrolysates of brain and liver of the newborn mouse after exposure for 24 hours *in vivo* and *in vitro* to glucose-U-C^{14}. Sky-Peck, Pearson and Visser ('56) have repeated the *in vitro* experiments with brain of the newborn mouse. The *in vivo* results on the liver of the first of these groups of investigators agree with ours with respect to the amino acids in protein which contain C^{14} derived from glucose-U-C^{14} except that we failed to find evidence of C^{14} in methionine and phenylalanine; in cortical protein, unlike their results with whole brain, we failed to find labeling of cystine, lysine, valine, methionine, arginine, leucine and isoleucine but did find C^{14} in proline. The low level of radioactivity in all of these amino acids except cystine and the longer exposure to higher levels of glucose-U-C^{14} in their experiments may well account for the discrepancies. In our experiments with both tagged glutamic acid and glucose, although certain essential amino acids were found to contain C^{14} (in the experiment with tagged glutamic acid, C^{14} was present in arginine, phenylalanine, leucine, isoleucine and threonine; with tagged glucose, in arginine and histidine), this was at such a low level that there is no conflict with the viewpoint of the indispensibility of these amino acids both in cortex and liver of the growing mouse. The *in vitro* studies referred to above have shown that under such conditions glucose-U-C^{14} contributes C^{14} to all amino acids of protein of brain except threonine and has led Winzler and his collaborators to emphasize the caution necessary in this field in transferring findings of *in vitro* experiments to the living animal.

Finally, it may be pointed out that it can be assumed by analogy to the rat (Flexner, '55) that the nerve cells of the mouse at birth are in the neuroblast stage. The observations that glutamic and aspartic acids are derived from glucose indicates that the citric acid cycle is functional in these immature cells as well as in the liver.

SUMMARY

Using glutamic acid, glucose and a mixture of phenylalanine, leucine and isoleucine, all uniformly labeled with C^{14}, estimates of the following quantities in the cerebral cortex and liver of the living newborn mouse have been made: (a) the rate at which the carbon of tissue glutamic acid and glutamine is derived from glutamic acid of the blood, (b) the rate at which the carbon of several non-essential amino acids of the tissues is derived from blood glucose, (c) the rate at which the carbon of certain essential amino acids of the tissues is derived from these amino acids in the blood, and (d) the rate at which the pools of these amino acids of the tissues are drawn upon for protein synthesis. The importance of glucose carbon for synthesis of glutamic and other non-essential amino acids is pointed out and is related to the low rate of incorporation of labeled glutamic acid in protein as compared to the essential amino acids. The most striking difference in behavior of liver and cortex is in the relatively high rate of turnover in most of the hepatic pools which have been studied.

ADDENDUM

Since the preparation of this paper, pertinent articles on the turnover of proteins of the brain by M. K. Gaitonde and D. Richter (Proc. Roy. Soc. B, *145:* 83, 1956) and A. Lajtha, S. Furst, A. Gerstein and H. Waelsch (J. Neurochem., *1:* 289, 1957) have come to our attention.

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THE HEMOLYTIC VOLUME OF HUMAN ERYTHROCYTES[1]

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ONE FIGURE

INTRODUCTION

The concept that erythrocytes swell osmotically to a definite volume prior to their hemolysis was first introduced by Jacobs ('27). This unique volume has become known as the hemolytic volume and is conventionally represented by the symbol, $\overline{V}_h$. Several separate approaches to the measurement of this quantity have been described (Castle and Daland, '37; Ponder, '37; Guest and Wing, '42). The present paper presents a new method in which the accuracy of the determination is considerably enhanced and evaluates certain environmental factors which influence the hemolytic volume. This method rests upon assumptions, different from those involved in any of the previous techniques, which in turn can be tested independently.

DESCRIPTION OF METHOD

Theoretical. The $\overline{V}_h$ of a red blood cell is defined as that volume the cell attains when the membrane becomes permeable to hemoglobin (Hb) with the subsequent loss of Hb to the external environment (hemolysis). When hemolysis is complete, the concentrations of Hb within the red blood cell ghosts and in the hemolysate are identical. This implies that the fraction of Hb remaining in the ghost (Hb_G) is equal to

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the ratio of the volume occupied by the ghosts to the total volume of the suspension. Thus,

$$(\mathrm{Hb_G}) = \frac{\sum_{i=1}^{n} (\mathrm{V_h})_i}{\mathrm{V}} = \frac{\mathrm{n\overline{V}_h}}{\mathrm{V}}$$

where n is the number of cells in the suspension; V, the total volume of the suspension (C + H) and $\overline{\mathrm{V}}_\mathrm{h}$, the average hemolytic volume.

The volume of packed cells, C, added to the hemolyzing solution (H) is equal to the number of cells times the average cell volume, $\mathrm{n\overline{V}_o}$. It follows that once C, V and $(\mathrm{Hb_G})$ are determined $\overline{\mathrm{V}}_\mathrm{h}/\mathrm{V_o}$ may be computed according to the formula:

$$\frac{\overline{\mathrm{V}}_\mathrm{h}}{\mathrm{V_o}} = \frac{\mathrm{V}}{\mathrm{C}}\,(\mathrm{Hb_G})$$

This ratio represents the average fractional increase in the volume of the cells at the time of hemolysis relative to their average initial volume. In practice, the fraction of hemoglobin in the hemolysate $(\mathrm{Hb_H})$ is measured and $\mathrm{Hb_G}$ determined by difference. Changes in the ratio of V/C do not affect the value of $\overline{\mathrm{V}}_\mathrm{h}$; however, if incomplete hemolysis is to be avoided this ratio should be no less than three. At high values of V/C the experimentally determined values of $\mathrm{Hb_H}$ are so close to unity that the errors in $\mathrm{Hb_G}$ are very large.

Example of method. In table 1 are presented the results of a typical experiment. Human blood was defibrinated and the cells packed at 3600 × G. The hematocrit was determined (93.2%) using the method of Parpart and Ballentine ('43). A known volume of packed cells was delivered using a modified Krogh pipette into a known, stirred volume of distilled water. After 30 minutes each mixture was rapidly diluted with 0.17 M NaCl, phosphate buffered (pH = 7.55) to a final volume of 100 cm.^{3} Aliquots were immediately removed, centrifuged and the optical density of the supernatants were read at 540 mμ with a Beckman spectrophotometer. This latter procedure was repeated again 50 minutes later. Column e gives the total Hb in the system. (The blank subtracted from row 5, is prepared by placing the same volume of packed cells

TABLE 1

Sample determination of the hemolytic volume ($\overline{V}_h$) of human erythrocytes at 23°C

See text for discussion.

	SAMPLES				
	a	b	c	d	e
(1) cm^3 packed cells	0.2	0.2	0.2	0.2	0.2
(2) cm^3 packed cells corrected by hematocrit (C)	0.186	0.186	0.186	0.186	0.186
(3) cm^3 distilled water	0.5	0.65	0.9	1.2	100
(4) Corrected volume of medium (H)	0.514	0.664	0.914	1.214	100
(5) Optical density following dilution and correction for blank	0.297	0.344	0.393	0.431	0.565
(6) Repeat of (5) 50 minutes later	0.304	0.345	0.392	0.431	0.567
(7) Fraction total Hb (Hb_H)	0.512	0.593	0.678	0.743	1.000
(8) $HG_G = 1 -$ fraction total Hb	0.488	0.407	0.322	0.257	—
(9) $\overline{V}_h = \frac{(1+3)\,(8)}{(2)} = \frac{(V)\,(Hb_g)}{C}$	1.78	1.79	1.80	1.78	—

directly into a final volume of 100 cm^3 of the NaCl-phosphate solution). The fraction of Hb in the supernatants of the test mixtures (columns a through d) are obtained by dividing each by the value in column e. Hb_G is given by one minus the values so obtained as shown in the table.

Validity of method. This rests upon the following three assumptions being met: (1) that 100% hemolysis is achieved; (2) that Hb proceeds to diffusion equilibrium—that is, equal concentrations inside and out—at the time of hemolysis and (3) that no rehemolysis (cf. Hoffman, '54) occurs.

The first assumption can be checked by centrifuging the initial mixture, removing a given volume of the supernatant and diluting to some convenient concentration to be determined spectrophotometrically. This is compared to a similar dilution of the mixture to which had been added a lytic agent[4] sufficient to insure complete hemolysis. When these values are compared the Hb concentrations are identical which would not be the case unless 100% hemolysis took place. When tested this was found to hold in all of the instances reported above. The correctness of the second assumption can be inferred from the fact that the $\overline{V}_h$'s so determined were independent of the ratios of V/C as shown in table 1. Parpart ('53) has previously demonstrated that Hb goes to diffusion equilibrium at all ratios of V/C where 100 percent hemolysis is insured. In addition, Hoffman ('54) measured directly the concentration of Hb in ghost and hemolysates at different ratios of V/C and found them at each ratio to be equal. These results are in marked contrast to those reported by Ponder ('42) and provide strong evidence that the concept of a bound Hb as developed by Ponder ('55) appears untenable at the present time.

Prevention of rehemolysis. The validity of the third assumption can be tested by studying the Hb concentration in the supernatant as a function of time following dilution as indicated in table 1. That no rehemolysis occurred is evident from the agreements between rows (5) and (6).

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[4] The agent used was isooctylphenoxypolyethoxyethanol obtainable as "Cutscum" from Fisher Scientific Co. The addition of small amounts of this substance renders an opaque mass of packed ghosts completely transparent, thus apparently solubilizing the ghost. Cotzias, *et al.* ('54) described the use of this agent to solubilize monoamine oxidase.

On the other hand, it was observed that if, instead of unwashed cells, washed cells were used some rehemolysis took place. This rehemolysis could be eliminated by adding a small amount of plasma (25 mm^3/cm^3) to the distilled water prior to the addition of washed cells. Dialysates of plasma and urine also prevented rehemolysis. A number of dialyzable plasma constituents were tested (HCO_3^-, $SO_4^=$, K^+, Mg^{++}, Ca^{++} and glucose) but only Ca^{++} (less than 2 mM/1) was found to be as effective as plasma in preventing all rehemolysis. Mg^{++}, adenosine, inosine, guanosine and hypoxanthine were found to substantially retard the rate of rehemolysis whereas adenine, cytosine, uridine and nicotinamide were without effect in preventing rehemolysis.

It was found necessary to have the plamsa or Ca^{++} present at the time of hemolysis; addition just prior to dilution or presence in the diluting solution alone did not prevent rehemolysis. In the experiments using washed cells reported below plasma was routinely added to eliminate rehemolysis.

RESULTS

Table 2 summarizes the $\overline{V}_h$ findings on the red cells from three normal male adults. Each experiment was performed on unwashed, defibrinated blood as described above and involved measurements at three to 6 separate ratios of V/C. The $\overline{V}_h$ is seen to vary between 1.54 and 1.75 depending upon the particular blood used in the determination. The differences in the values found for the three bloods are statistically significant ($P = 0.01$ or less) and therefore must indicate individual variation. Listed also in table 2 are $\overline{V}_h$ values for erythrocytes derived from two pathological bloods. It is apparent that there is considerable difference in the $\overline{V}_h$ obtained for these two types of blood and normal blood.

The typical effects of temperature and tonicity on the measured $\overline{V}_h$ are shown in figure 1. The time allowed for prior equilibration of the unwashed defibrinated cells at each temperature was one hour. The cells used in the tonicity measurements (at 23°C) were washed 3X with NaCl-phosphate buffered at pH 7.3. The results show that the $\overline{V}_h$ is directly proportional to both the temperature and to the reciprocal of the tonicity. Since the $\overline{V}_h$ is actually the ratio $\overline{V}_h/V_o$, the observed influence of temperature (Jacobs and Parpart, '31) and tonicity (Lucké and McCutcheon, '32) is obviously on V_o. Thus, an increase in tonicity or temperature would decrease V_o and increase the apparent $\overline{V}_h$. From the known equivalent of the isotonicity of plasma the $\overline{V}_h$ may be interpolated from the appropriate curve in figure 1 and compared with the measured $\overline{V}_h$ for unwashed cells performed as a part of the same experiment (see Green and Hoffman, '53). The interpolated value is approximately 1.55 and the measured value is 1.51 in reasonably good agreement. This means that there is no observed effect on the hemolytic volume when cells are suspended in a saline-phosphate mixture isotonic with plasma (cf. Ponder, '37). An analogous argument would apply to the effects of temperature.

TABLE 2

The hemolytic volume ($\overline{V}_h$) of normal and certain abnormal human erythrocytes at 23°C

The mean $\overline{V}_h$ value is given together with its standard error.
The values in parentheses represent the range of variation.

INDIVIDUAL	DESCRIPTION	NO. OF EXPTS.	$\overline{V}_h$
A	Normal	3	1.75 ± 0.02
B	Normal	6	1.68 ± 0.03
C	Normal	6	1.54 ± 0.03
D[1]	Sickle cell anemia	1	2.35 (± 0.11)
E[2]	Hereditary spherocytosis	2	1.12 (± 0.12)

[1] S-S hemoglobin.

[2] Eight years prior splenectomy.

The hemolytic volume may be measured under circumstances in which hemolysis takes place in solution of penetrating solutes. For instance it was found that the $\overline{V}_h$ was not altered when the cells were hemolysed by 0.3 M glycerol, 0.3 M monoacetin or 0.17 M NH_4Cl. On the other hand, measurements of $\overline{V}_h$ for erythrocytes in which hemolysis is induced by lytic agents, e.g., n-butanol, have been unsuccessful since no way of preventing rehemolysis has yet been found. In this instance, the addition of plasma or Ca^{++} was without effect.

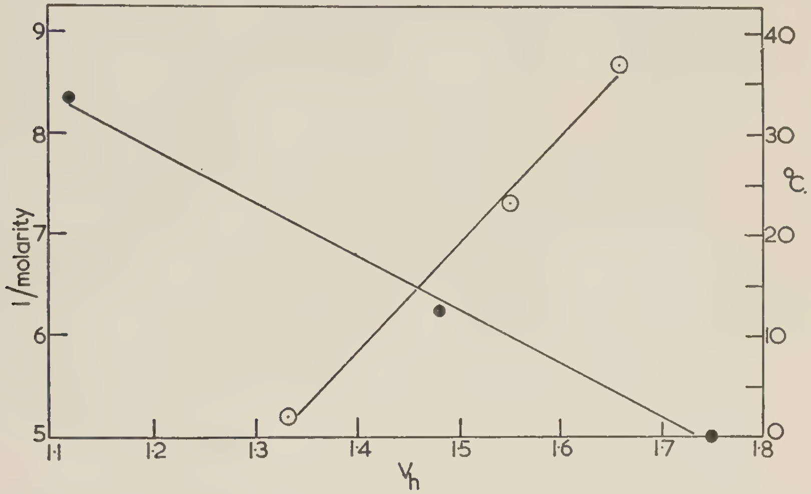

Fig. 1 The influence of temperature and tonicity on the hemolytic volume ($\overline{V}_h$) of normal human erythrocytes. Symbols: ● for ¹/molarity (left ordinate); ⊙ for temperature in °C (right ordinate).

DISCUSSION

The values for the $\overline{V}_h$ of human red cells presented above are in good agreement with the results obtained by previous workers and summarized in table 3. The methods used by Castle and Daland ('37) and Guest and Wing ('42) assume that the volume of the sphere prior to hemolysis is identical to the hemolytic volume. One method was to calculate the volume of a sphere which had the same surface area as the disc from which it was derived; a second method utilized the Boyle-Van't Hoff law to extrapolate to the volume the cells were at when 50 percent hemolysis had occurred. In another method, described by Guest and Wing ('42), the $\overline{V}_h$ is estimated from a determination of the volume of the remaining cells by hematocrit for different degrees of hemolysis. By measuring at the same time the percent hemolysis the hematocrit can be appropriately corrected for the lost cells. The first method is confronted with the uncertainties involved with the measurement of the surface area of the initial disc (cf. Ponder, '48a); the latter two methods are encumbered primarily by the assumptions of the relationship between the percent hemolysis and the hemolytic volume.

TABLE 3

Summary of previous measurements of the hemolytic volume ($\overline{V}_h$) of human erythrocytes

REFERENCE	CALCULATED FROM SURFACE AREA	BOYLE-VAN'T HOFF EXTRAPOLATED VALUE	HEMATOCRIT CORRECTED VALUE
Castle and Daland ('37)	1.59	1.75 (1.51–1.67)[1]	—
Guest and Wing ('42)	1.71–1.76	1.70–1.79	1.72–1.80

[1] Values approximate only. Taken from their figure 2.

The method described in this paper obviates the above difficulties since the volume that is actually measured is the volume at which time the cells become permeable to Hb, i.e., the hemolytic volume. The fact that all of these methods yield quite similar results support the hypothesis (see Jacobs, '27) that the cell reaches its hemolytic volume without a perceptible increase in surface area. Hemolysis, *per se,* would then be associated with the instability induced by a slight increase in the surface area of the cell membrane. This view is supported by the experiments reported by Ponder ('48b).

Given that the volume of the sphere, prior to hemolysis, can be equated to the hemolytic volume, a recalculation of the surface area of the normal cell becomes possible. Thus, using an initial volume equal to 87 μ^3 and an average hemolytic volume of 1.65 the computed surface area is approximately 133 μ^2. This value differs significantly from 163 μ^2 given by Ponder ('48a). It should be emphasized, however, that the individual differences in the $\bar{V}_h$ found in the present work would accordingly modify this calculation.

The hemolytic volumes found for red cells derived from sickle cell anemia and hereditary spherocytosis (as well as from normal) are consistent with the interpretation of Heden ('34) and Castle and Daland ('37) that the values so observed are reflections of the diameter to thickness ratio of the initial disc. This ratio would thus indicate the osmotic differential necessary to make the cells swell to their hemolytic volume.

It is of interest that the prevention of rehemolysis noted above is dependent upon the so-called reversal of hemolysis (Bayliss, '24) brought about by dilution with isotonic saline. The ghosts derived from such a treatment display a considerable number of characteristics not unlike those shown by intact cells (cf. Hoffman and Tosteson, '56).

SUMMARY

1. A new method is described for measuring the hemolytic volume ($\bar{V}_h$) of human erythrocytes. The assumptions underlying the method were tested experimentally and found to be valid.

2. The $\bar{V}_h$ of normal erythrocytes was found to be constant for each individual but was found to vary from 1.54 to 1.75 (times their initial volume) for different individuals. The $\bar{V}_h$ of erythrocytes, derived from patients with sickle cell anemia and hereditary spherocytosis were 2.35 and 1.12, respectively.

3. Some environmental factors which can influence the $\bar{V}_h$ are evaluated and certain implications of the results are discussed.

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ON THE RELATIONSHIP OF CERTAIN ERYTHROCYTE CHARACTERISTICS TO THEIR PHYSIOLOGICAL AGE [1]

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ONE FIGURE

GENERAL INTRODUCTION

Typically, the differential response of erythrocytes to a wide variety of conditions is referable to inherent differences among the erythrocytes comprising that population. The experimental dissection of a population has essentially been (see below) of two types: isolation of different portions of the population for separate study or labeling a certain fraction of the population and observing that fraction within the whole population. The present communication deals with both of these approaches and, in particular, describes techniques for relating certain erythrocyte characteristics to their physiological age.

MATERIALS AND METHODS

Of fundamental importance to this work is the observation of Hahn, et al. ('41) that Fe^{59} may be incorporated into the hemoglobin (Hb) of the newly forming red cells and can, within certain limitations, serve as an indicator of their age. Approximately 0.1 millicurie Fe^{59} ($FeCl_3$ as carrier) in isotonic buffered saline was injected intraperitoneally into normal adult white rabbits. Radioactivity was measured with a well-type scintillation counter employing a photomultiplier tube. The standard error of the counting rate was less than ±1%.

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[1] This work was supported by the Atomic Energy Commission and the Whitehall Foundation.

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Heparinized blood obtained from the cut ear (2–3 cm^3) was used for the density and osmotic resistance determinations described below. For the experiments dealing with the hemolytic volume (V_h) and hemolysis studies other than osmotic resistance, blood was obtained from the heart. The blood used for the V_h determination was defibrinated. All experiments were performed at room temperature.

The procedure used for the relative cell density measurements was a modification of that described by Chalfin ('56). Approximately 1.5 cm^3 of whole blood were centrifuged for 40 minutes at 15,000 XG in an air turbine (Parpart and Ballentine, '43). The plasma, containing negligible radioactivity, was removed. The packed mass of cells was divided into 5 equal layers, separated with pipettes, diluted to a total volume of 3 cm^3 with distilled water, thoroughly mixed and then counted.

Osmotic resistance was determined in the following way. 0.05 cm^3 of blood was delivered, using a modified Krogh pipette, into each of a series of tubes containing 20 cm^3 of NaCl–PO$_4$ (pH = 7.4) of different concentrations (cf. Parpart, et al., '47). After 30 minutes, hemolysis was stopped by the addition of concentrated NaCl. The tubes were then centrifuged at about 10,000 XG for 5 minutes. Three cm^3 of the supernatant were removed and counted. The Hb concentration of the same supernatant was determined at 540 mμ with a Beckman spectrophotometer. Thus the per cent hemolysis and the per cent of the total Fe^{59} activity of the supernatant could be appropriately calculated.

Relative density and age. Table 1 shows the distribution of Fe^{59} labeled red cells in the different levels of centrifuged samples of blood. The different samples of blood were obtained at the indicated intervals of time following an initial single injection of Fe^{59}. The maximum activity appears in level 1 at two days, shifts to level 2 by 15 days, to level 3 by 26 days and to level 5 by 38–50 days. A plot of the position of the maximum activity against time indicates that this transition is approximately linear up to 45–50 days. It has been observed by Chalfin ('56) (see also Keitel, '55) that rabbit reticulocytes, identified by the standard staining techniques, are less dense than the older cells and are concentrated at the top of a centrifuged sample of cells. The data presented in table 1 corroborate this finding, but show further the sequential changes in relative density of the remaining cellular population as they continue to age.

TABLE 1

Relative cell density as a function of physiological age

Levels 1 through 5 represent division of a centrifuged column of red cells from the top to the bottom, respectively

DAYS FROM INJECTION	PERCENT OF TOTAL Fe^{59} ACTIVITY IN LEVELS				
	1	2	3	4	5
2	63.1	18.5	10.5	6.1	1.8
5	59.3	23.8	11.2	4.0	1.7
15	25.7	30.0	24.4	13.6	6.3
26	14.3	19.7	24.9	21.4	19.7
38	9.4	13.7	23.3	24.9	28.7
48	10.9	11.8	20.5	25.0	31.8
55	13.1	16.5	20.4	25.2	24.8
62	18.2	16.2	19.1	25.8	20.7

It will be noted that after the fifth day no level in the tube exhibits an activity representing a major fraction of all the tagged cells in the sample. A number of factors may contribute to this dispersion. (1) The levels may not have been separated cleanly. (2) Centrifugation time may have been too short. (3) The age distribution may have been altered by the steady flow of new tagged cells into the population (see below). (4) A fraction of the tagged population may not exhibit uniform behavior relative to the properties of age and density. These qualifications cannot be evaluated adequately without further study.

Relative age and osmotic resistance. Figure 1 shows the relationship of the pattern of osmotic resistance of the cells containing Fe^{59} to the pattern exhibited by a sample from the whole population. The determinations were performed on aliquots of the same blood used in the density studies described above. The method of plotting the per cent hemolysis against the per cent of the total Fe^{59} present in the supernatant facilitates comparison of the two types of data. It can be seen that the young cells (i.e., 5 days after injection)

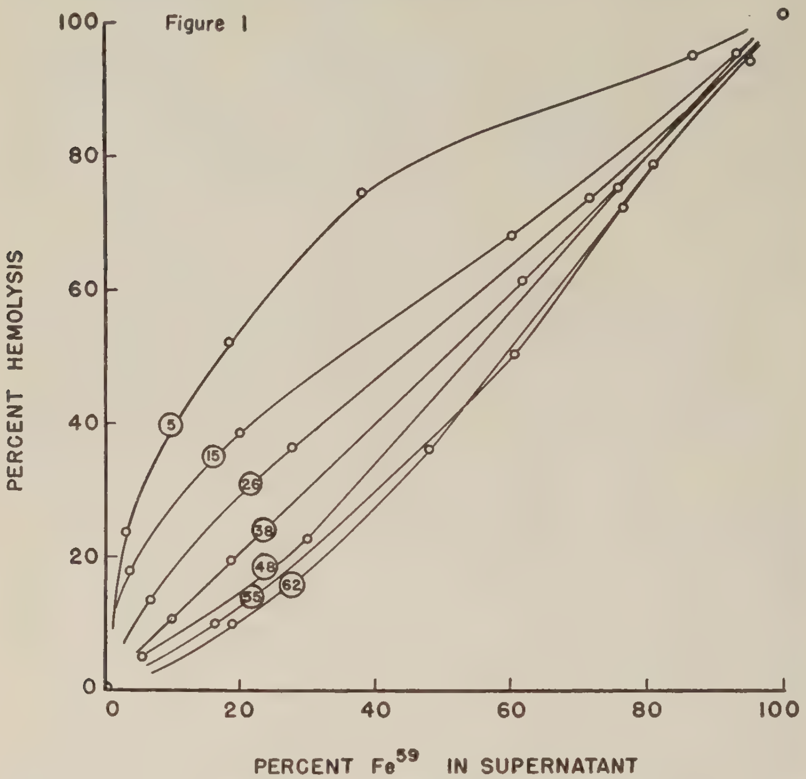

Fig. 1 Osmotic resistance as a function of physiological age. The ordinate represents the percent hemolysis obtained using Hb as the indicator. The abscissa is the percent of the total Fe^{59} activity appearing in the same supernatants. The numbers in the circles indicate the days following the initial injection of Fe^{59} the blood was tested.

are the most resistant to osmotic hemolysis. As the cells age their osmotic resistance decreases. The curve for 38 days shows that the group of cells containing Fe^{95} exhibit a pattern of osmotic resistance indistinguishable from that of the majority of cells in the population. As the Fe^{59} labeled cells continue to age they become less osmotically resistant. These results indicate that the resistance to hemolysis is correlated directly with the relative age of the cells comprising the whole population. The finding of Chalfin ('56) of an osmotic resistance difference between reticulocytes and the "bottom layer" cells is, thus, confirmed. In addition it has been shown that the mature red cell suffers an increase in fragility with age.

DISCUSSION

The interpretation of the data presented is limited by the continuous appearance of new tagged cells in the whole population due to the gradual incorporation and reutilization which attends the use of Fe^{59} as a tracer.[3] For instance, inspection of each column in table 1 reveals an orderly fluctuation of the per cent Fe^{59} activity with time in each particular level, e.g., the decrease in the per cent Fe^{59} activity in level 5 after 48 days shows that the most dense and oldest cells are the most likely ones to be removed from the circulation and that this is reflected in level 1. Further, the displacement of the curves for 48, 55 and 62 days seen in figure 1 toward slope $= 1$ (38 day curve) also represents the influence of the above operating factors.

The procedure used for the determination of osmotic resistance is in principle the same as that described by Stewart et al. ('50) (see also Cruz et al., '41) in similar studies on dog erythrocytes. However, the results they reported differ from those presented above in two important aspects. The first is that in contradistinction to rabbit cells the correlation with age is reversed, i.e., young dog erythrocytes are less osmotically resistant than older cells. Second, the cells containing Fe^{59} did not distinctly become the most resistant cells, i.e., the tagged cells did not "pass through" the whole population response. These considerations obviously emphasize species differences in the mechanism of aging of their respective red cells.

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[3] Normalizing for decay of Fe^{59} and correcting, in turn, for the amount of blood lost, a relative specific activity (RSA) can be calculated from the data accumulated for the 100% points represented in figure 1. When the RSA is plotted against time the resultant curve could be superimposed upon the corresponding curve for rabbit presented by Brown and Eadie ('53). The constant for indiscriminate destruction, k, computed from the above data was found to equal 0.0088, in good agreement with the results reported by Brown and Eadie ('53).

The method used to study osmotic resistance can be adapted in like manner to study the response of tagged cells relative to the whole population to other forms of hemolysis. For instance, in table 2 are indicated the results obtained when the cells of the 62nd day sample are hemolyzed with thiourea and with n-butyl alcohol. It can be seen that hemolysis with n-butyl alcohol is correlated with the relative age of the cells, whereas hemolysis with thiourea appears to be independent of the age of the cells. In other words, the permeability of cells to thiourea appears to be the same throughout their lifetime but their susceptibility to lytic agents changes with time. Thus, this method offers the possibility of determining whether or not the permeability constants of a cell for a series of compounds (e.g., non-electrolytes, see Jacobs and Parpart, '35) is independent of the age of the cell. Analogous

TABLE 2

Dependence on the method of hemolysis in obtaining differential response of tagged cells relative to the whole population

The blood used was the sample collected on day 62 referred to in table 1 and figure 1

METHOD OF HEMOLYSIS	PERCENT HEMOLYSIS FOR 50% Fe^{59} IN SUPERNATANT
Osmotic resistance	37.5
Butanol	35.0
0.3 M Thiourea	47.5

measurements would be helpful in analyzing the action of various agents (cf. Ponder, '48).

For an experiment such as suggested above, it would be necessary to know the hemolytic volume (V_h) of the cells in computing the permeability constants (Jacobs, '55). This can be done using the method described by Hoffman et al. ('58). In addition to using Hb as an indicator for the determination of the V_h of the whole population the Fe^{59} activity can similarly be used to calculate the average V_h of the tagged cells. The result of one such measurement[4] yields a value $V_h = 1.49 \pm .04$ for the whole population and $V_h = 1.62 \pm .05$ for the cells containing Fe^{59}. This implies that the V_h of cells changes with age.

Chalfin ('56) found that the amount of potassium per cell appeared to decrease as the cells grew older, but that the concentration of potassium per cell remained the same (cf. Parpart, et al., '51). On the other hand, the amount of Hb per cell was found to be independent of the age of the cell. Further, the reticulocytes were found to have larger volume and to possess a different thickness to diameter ratio than the older cells. The results reported in this paper, gleaned from the blood of a normal animal unencumbered by any hematological stress, support the conclusion reached by Chalfin ('56) that red cells decrease in volume essentially at the expense of a net loss of potassium. This is shown by the fact that relative cell density is correlated with age and that the osmotic resistance (also correlated with age) reflects differences in cellular shape (Castle and Daland, '37). It must be emphasized that the characteristics studied in this paper describe changes which accompany aging and do not necessarily focus on the underlying mechanism.

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[4] This measurement was performed on a rabbit which had been injected 30 days previously with Fe^{59} as described in the section on *Materials and Methods.* The percent Fe^{59} activity (analogous to table 1) in the separated levels (1 through 5) of a centrifuged portion of blood was 29.7, 23.2, 23.8, 14.6 and 8.8, respectively. The fact that the relative specific activity of the supernatant was the same for each ratio of volume of hemolyzing water to volume of cells used in the determination of the V_h served as an additional indicator for the occurrence of 100% hemolysis.

SUMMARY

Certain characteristics of rabbit red cells labeled with Fe^{59} were studied as a function of their physiological age. It was found that the relative cell density increases as the cells increase in age; the osmotic resistance decreases as the cells increase in age. Methods are indicated for studying other properties of red cells which may be related to their age. The characterization of the aging process of red cells is discussed.

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THE ACTION POTENTIAL OF FROG NERVE AS AFFECTED BY TEMPERATURE, NARCOSIS, AND STIMULATION FREQUENCY[1]

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SIX FIGURES

While the effects of temperature on nerve conduction have been studied by several investigators, there is little in the way of explanation that can be offered for the results obtained. Gasser ('31) reported the temperature coefficient of the action potential of frog nerve to be large and positive. His observations indicated that the spike declined sharply below 15–20°C with conduction failing in the neighborhood of 5°C. Schoepfle and Erlanger ('41), working on single fibers in a small branch of the peroneal nerve of the frog, found the temperature coefficient of the spike to be small and negative. Cooling from 25° to 8°C increased the spike amplitude by a factor of 1.2. Lorente de Nó ('47) observed that when the temperature of frog nerve is lowered from 18°C, the spike height first increases and, after passing through a maximum at about 10°C, progressively decreases. Hodgkin ('37) noted that cooling a nerve prolongs its absolute refractory period and thus "acts as a block to the second but not to the first of two impulses." The maximum increase in absolute refractory period was about 50 msec. Hodgkin and Katz ('49), working with squid giant fibers, found that cooling decreased the slope of the descending limb of the spike much more than that of the ascending limb; lowering the temperature from 32° to

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[1] Aided by a grant (B-139) from the National Institute for Neurological Diseases and Blindness, Bethesda, Maryland.

10°C resulted in a progressive increase in spike amplitude. Wright ('55) observed a loss of repetitive firing of single crustacean motor axons when the normal absolute refractory period of 1 msec was increased to 25 msec at 5°C. Thus there is some agreement as to the parameters of nerve function most affected by changes in the ambient temperature; low temperatures affect the refractory period more than either the duration or height of the spike. The increased duration of the spike at low temperatures appears to be largely the result of a slowed rate of increase in potassium conductance of the membrane, and, in general, low temperatures appear to affect primarily the recovery processes of the fiber.

The resting membrane potential is also significantly affected by temperature changes. Lorente de Nó ('47) reported a marked drop in membrane potential for frog nerve below 6°C, in contrast to results with muscle, while Hodgkin and Katz ('49) observed a significant fall in resting potential of squid fibers above 20°C, but not at low temperatures.

The present study is concerned with the relationships that exist between the properties of frog nerve at low temperatures, where conduction is blocked, and nerve where conduction is blocked by chemical agents.

METHODS

A lucite chamber was constructed so as to allow a short length (ca. 6 mm) of frog sciatic nerve about 1 cm beyond the stimulating electrodes to be brought to any temperature desired by water circulating in a stainless steel tube as shown in figure 1. The temperature of the nerve at the point of cooling was measured using a copper-constantan thermocouple and the thermal voltages were amplified by a D. C. chopper amplifier and displayed on one channel of a dual beam oscilloscope, while the other channel was used to record the action potentials obtained from electrodes about 1 cm beyond the point of cooling via a RC coupled amplifier. When temperatures above the ambient temperature of 22°C were employed, the whole chamber was heated. The entire nerve was therefore subjected to temperature elevations. Some narcotics were applied locally to the cooled region only, while others were introduced into the gas phase in the chamber with 95% O_2. Stimulation was effected with 0.2 msec square wave pulses and was supramaximal for A-fibers in all cases.

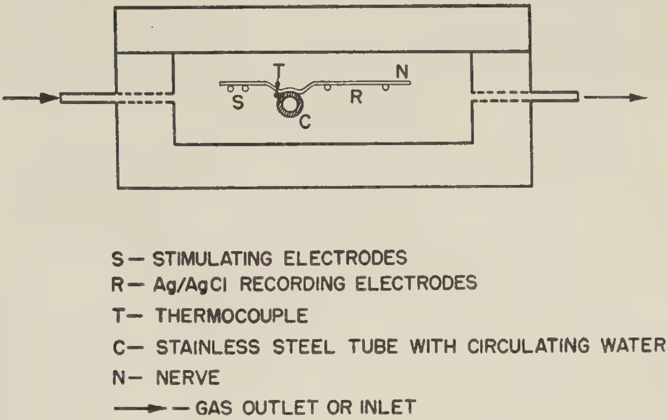
T
N
S
R
C
S — STIMULATING ELECTRODES
R — Ag/AgCl RECORDING ELECTRODES
T — THERMOCOUPLE
C — STAINLESS STEEL TUBE WITH CIRCULATING WATER
N — NERVE
→ — GAS OUTLET OR INLET

Fig. 1 Diagram of apparatus used to provide temperature variations.

RESULTS

Figure 2 shows the temperature dependence of the action potential of a normal frog sciatic nerve when stimulated at a frequency of 30/sec. As nerves are allowed to stand at 4°C in frog Ringer solution, the low temperature limb of the curve shifts toward higher temperatures. After 10 hours, this part of the curve is shifted about 8°C. The region between 10° and 38°C has a uniformly low positive temperature coefficient, the Q_{10} is about 1.1.

The length of nerve cooled in these experiments was about 6 mm, this is the minimum length which must be blocked in order to prevent electrotonic spread across a block (Kato, '24).

The observed inter-relation between spike height, temperature, and frequency of stimulation is in part explicable in terms of processes known to occur in nerve although a quantitative relationship cannot be obtained between spike height and the number of fibers responding to a stimulus. Cooling a nerve is known to result in a prolongation of the refractory period (Hodgkin, '37; Wright, '55) and the relative refractory period has been observed in these experiments to increase to 100 msec at 5°C. This marked prolongation of refractory period obviously sets a limit to the frequency response of the individual fibers. Failure of conduction for impulses separated from each other by more than 100 msec has been observed at 6°C and this effect cannot be accounted for by refractoriness of the fibers. When a nerve is narcotized, the amplitude of the action potential of the individual fibers is reduced and a critical reduction in spike amplitude exists where conduction fails because of insufficient electrotonic spread (Davis et al., '26; Tasaki, '39). The compound action potential can thus be expected to show changes in amplitude because of changes in the number of fibers responding. The experiments reported here cannot discriminate absolutely between these two types of change but those observed are consistent with the view that (a) from 16° to 6°C, the decline in compound spike height with frequency is a result of a pro-

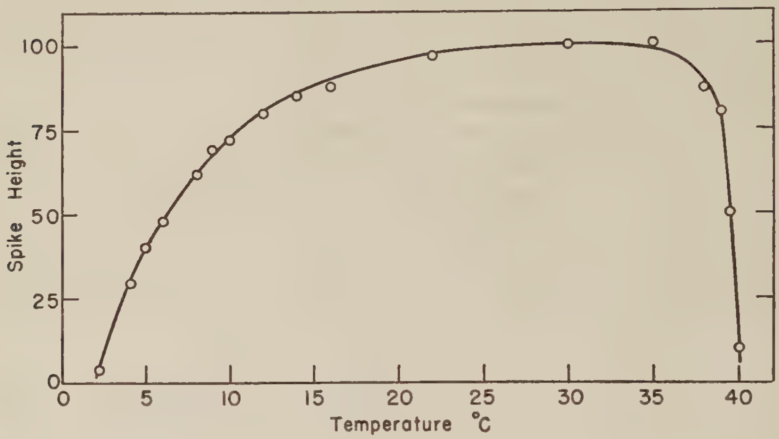

Fig. 2 Temperature dependence of the spike amplitude obtained with normal frog sciatic nerve. Relative spike height is plotted versus the temperature. The response at 30°C is taken as 100%. Stimulation is supramaximal at 30/sec.

longation of the refractory period of most of the fibers, and (b) at 6°C and below there is a change in the membranes of most of the fibers such that a frequency independent conduction block occurs. In a multifibered preparation there must exist some dispersion in temperature over which a specified prolongation of the refractory period of the fibers will occur, and also some dispersion in the frequency independent temperature at which blocking of conduction will take place.

To relate the decline in spike height occurring in these experiments to the number of individual fibers failing to respond is complicated by the non-linear relation which exists between compound spike amplitude and number of fibers responding. Gasser and Erlanger ('27) found that the compound spike amplitude varies linearly with the cross sectional areas of the individual responding fibers, fibers with greater diameters making greater contributions to the over-all spike potential. Thus a 50% reduction in the spike height may indicate the loss of enough fibers to constitute 50% of the total conducting cross section, rather than a 50% reduction in the number of responding fibers. Also, it was not possible with the lengths of nerve employed to obtain sufficient temporal dispersion for a study of differential sensitivities to cooling of the various fiber classes. Some evidence was obtained that the β fibers are more sensitive to cold blocking than are the α fibers. Even though it is difficult to relate the observations, quantitatively, to individual fiber responses, it should be possible to compare the effects of temperature on normal nerve with those on narcotized nerve.

The blocking action of 0.2% novocaine at various temperatures is shown in figure 3. These experiments were done in two ways: the nerve was equilibrated with novocaine at various temperatures and the extent of the block measured after equilibrium was established (this was 85% complete in 20 minutes). The temperature was varied in nerves where the blocked region corresponded to the area that was in contact with the cooling tube and it was observed that a rise in temperature would rapidly relieve a block to the extent shown in figure 3. It should be emphasized that the effects of temperature variations after the drug had already reached an equilibrium in the nerve are rapid, occurring within a few seconds while the partitioning of the drug to the nerve requires a period of 20 minutes.

In these experiments, the slower fibers were most susceptible to the blocking action of novocaine. As with cooling, the β fibers seemed to always become blocked before the α fibers.

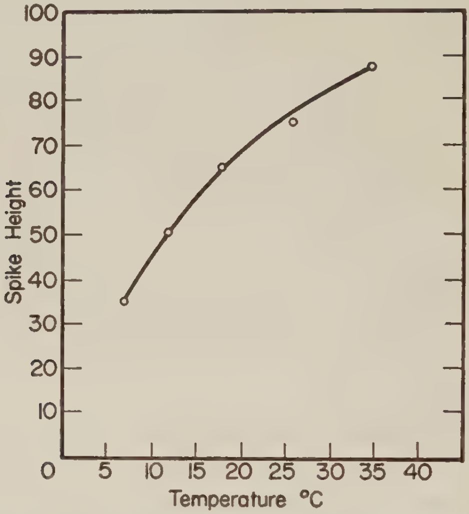

Fig. 3 Blocking action of .2% novocaine at different temperatures. The relative spike height observed is plotted against the temperature. The spike of the unblocked nerve at 20°C is taken as 100%.

Heinbecker and Bishop ('35) report that the anesthetic effects of procaine develop earlier with C than with A fibers. Gasser ('43) observed the same to be true with cocaine.

The use of hydrocarbons as blocking agents is desirable from the standpoint of the theory of narcosis because of the simpler nature of the forces of interaction between such molecules and the excitable mechanism of the nerve fiber. In addition it is desirable to use molecules that are "rigid" (e.g., where free rotation around carbon-carbon bonds is either not possible or where no shape change results if such rotation is possible) in order to specify a precise value for the size of the molecule. Benzene is such a molecule but preliminary experimentation with this substance showed that while it blocked conduction readily, raising the temperature did not relieve the block it produced to the extent shown by novocaine. To obtain a larger molecule, mesitylene (1,3,5-trimethyl benzene) was used. This molecule is apparently at the upper limit of size of substances that will enter the nerve membrane to the extent necessary to produce a conduction block because napthalene, which is only a little larger, would not produce a block even when applied for several hours.

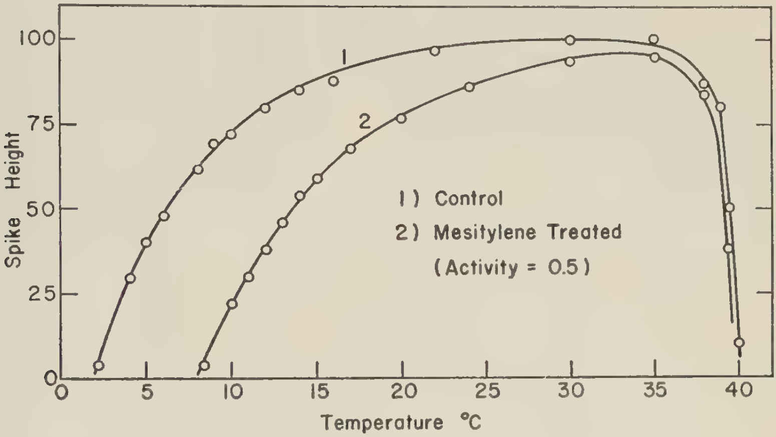

Fig. 4 Temperature dependence of the spike amplitude of nerve exposed to half-saturated mesitylene vapor for 20 min. compared with that of normal nerve. The curves shown are constructed from the averaged data of 12 experiments. The amount of variation observed can be seen by referring to table 1. Stimulation frequency = 30/sec.

Mesitylene was applied to the nerve as the half saturated vapor (thermodynamic activity = 0.5).[2] In each experiment one of the nerves of a pair was used as a control and was subjected to the same experimental procedure, with no mesitylene present. Mesitylene was administered for a period of 20 min. during which time the nerve was maintained at a temperature of 22°C and stimulated at the rate of 3/sec. At the end of this period, the action potential obtained at different temperatures was studied employing supramaximal stimulation at a rate of 30/sec. The data from 12 consecutive experiments are shown in figure 4 and table 1. The curves in figure 4 are averages constructed from the data obtained in the 12 experiments. The degree of variation and reproducibility of the experiments are shown in table 1 which gives the spike amplitude observed at 10°C for each mesitylene treated nerve along with that for the corresponding control nerve. Also shown for each nerve, is the temperature at which a block of 95% or greater occurs. Figure 4 indicates the marked change in temperature sensitivity which takes place when a nerve is treated with mesitylene in the above manner. Mesitylene has increased the temperature coefficient of the spike several fold in the range where it is normally low, or, alternatively, it has

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[2] The flow of mesitylene within the chamber was not subject to any significant temperature change. The mesitylene is, therefore, supplied at constant activity.

TABLE 1

Effect of cooling on mesitylene treated nerve[1]

	PERCENT OF 30°C CONTROL SPIKE AMPLITUDE OBSERVED AT 10°C			TEMPERATURE °C AT WHICH LESS THAN 5% OF 30°C CONTROL SPIKE AMPLITUDE OCCURS		
Experiment No.	Mesitylene Treated	Control	Difference	Mesitylene Treated	Control	Difference
1	20	75	55	9.0	3.0	6.0
2	36	75	39	7.5	2.5	5.0
3	12	66	54	8.5	2.5	6.0
4	26	78	52	8.0	1.5	6.5
5	7	60	53	9.5	3.5	6.0
6	3	70	67	10.0	2.5	7.5
7	23	75	52	8.5	2.5	6.0
8	20	80	60	8.5	2.5	6.0
9	40	87	47	7.5	0.5	7.0
10	12	67	55	8.5	2.0	6.5
11	20	55	35	8.5	3.0	5.5
12	45	80	35	7.0	1.0	6.0
MEAN ± Std. dev.	22 ± 13	72 ± 9	50 ± 10	8.4 ± .9	2.2 ± .9	6.2 ± .7

[1] Mesitylene administered for 20 min. as the half-saturated vapor.

shifted the low temperature limb of the spike height vs. temperature curve 6° in the direction of higher temperatures, a change similar to that produced by allowing nerves to stand in Ringer solution for 6 to 18 hours. Another point to be noted is the relatively small effect of mesitylene on the nerve at temperatures around 30°C.

Benzene, at an activity of 0.5, totally blocks the nerve readily at room temperature. At lower concentrations, it increases the temperature coefficient of the spike but not nearly to the extent shown by mesitylene. Lower temperatures (< 8°) are required to completely block benzene treated nerves, as compared with mesitylene or novocaine. The effects of benzene are rapidly reversible with washing, the temperature behavior of the nerve returns to normal as the compound leaves the fibers, while mesitylene effects are not reversible, the increased temperature dependence remains as a permanent property of the nerve.

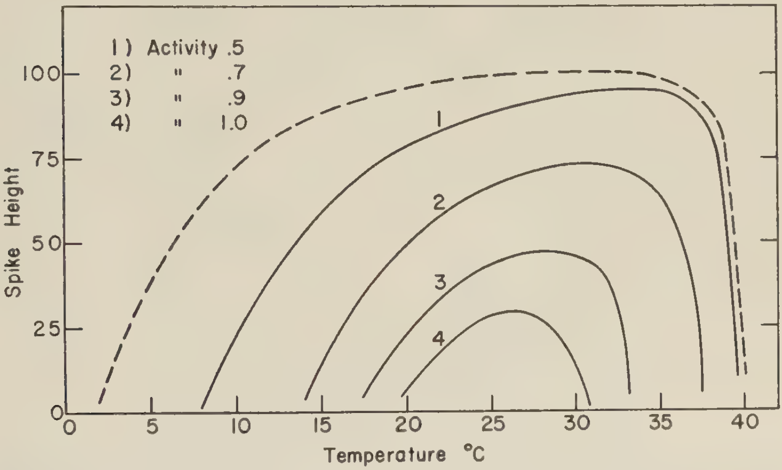

Fig. 5 Administering mesitylene vapor to the nerve at higher fractions of its vapor pressure results in the family of curves shown. The dashed curve indicates the behavior of normal nerve.

Higher concentrations of mesitylene yield the curves shown in figure 5; the nerves are blocked at higher and higher temperatures and tend to acquire an optimal temperature for conduction, above and below which blocking is enhanced.

The frequency at which the nerve is stimulated was observed to markedly influence the results obtained with both narcotized and cooled preparations. Figure 6 illustrates the frequency response of the mesitylene-treated nerve at various temperatures as compared with normal nerves. There is a direct parallel between temperature and frequency response, nerves become more frequency dependent as they become more temperature dependent without exception, above 6°C. The change in frequency response of the nerve with temperature is referable to a marked change in refractory period that takes place. Measurements with the double shock technique showed that, as previously observed by Hodgkin and by Wright (loc. cit.) cooling prolongs the refractory period. The absolute refractory period of frog nerve was increased from

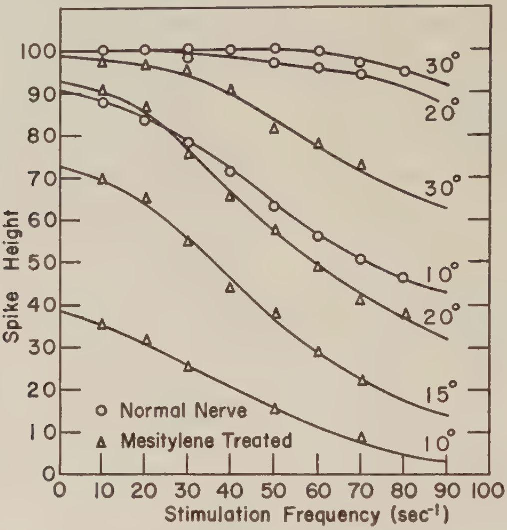

Fig. 6 Effect of stimulation frequency on mesitylene-treated nerve at different temperatures compared with the behavior of normal nerve. Shown are the averaged results of five experiments. The mesitylene was administered at an activity of 0.5 for a period of 20 min. It is apparent that raising the temperature of the mesitylene-treated nerve tends to make its behavior more normal.

a normal of 1, to 3 msec at 8°C and the relative refractory period from 3 to over 30 msec. Mesitylene, acting at room temperature, increased the relative refractory period to 9 msec, and cooling such a nerve to 15° increased this value to over 30 msec. Further cooling rapidly increased the refractory period to about 100 msec, a change produced in normal nerve by cooling to below 5°C.

DISCUSSION

It is evident that a conduction block in frog sciatic A-fibers can be obtained by the action of certain chemically inert substances acting at 20°C, or by a decrease in the temperature of the untreated nerve to about 4°C. These two treatments appear equivalent in their underlying mechanism since their effects are additive over a considerable range of temperature or narcotic concentration. The marked prolongation of the refractory period that is also brought about by either of the above treatments, or their action in concert, argues for a change in the rate at which ion permeabilities can be varied to produce the spike. Cooling or narcotizing a nerve can be thought of as analogous to increasing the forces of interaction between those elements of the membrane structure that are responsible for the permeability changes, and thus increasing first the time required for the membrane to reassume its initial condition and finally producing a sharp transition where the permeability changes of excitation can no longer take place, no matter how long the interval between test shocks may be.

The change of temperature that is necessary to reduce the spike height by 50% is only about 2°C in some cases and the sharpness of this response requires some comment.[3] While chemical reactions do have surprisingly large activation energies, it seems much easier to suppose that excitable membranes can undergo reversible phase transitions with small variations in temperature and that this mechanism underlies the phenomena that have been reported. By analogy with physical systems, the solid membrane is unexcitable, the membrane just above the transition temperature is viscous, and the addition of narcotics of certain sorts to the membrane promotes a phase transition at a higher temperature.

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[3] In some preliminary experiments with mesitylene-treated squid giant axons, the temperature change necessary to block conduction appears to be considerably less than 1°C. The transition temperature was about 13°C, although conduction cannot normally be abolished in squid axons at temperatures above 0°C. Regarding the sharpness of these effects, it is interesting to note that Hodgson and Roeder ('56) report significant changes in the response of single chemoreceptor cells when cooled by as little as 0.1°C.

Kirkwood ('54), in a theoretical discussion, treats the nerve membrane as a surface phase rather than a macroscopic membrane phase, and suggests that the impulse is a propagated phase transformation occurring in the surface phase. The propagated physical change in surface structure is responsible for the alterations in ionic permeabilities that are observed. The refractory period might then be considered as the time required to reform the original metastable phase.

The treatment above differs only in detail from that offered as an explanation for the results that have been reported in that the process of propagated excitation by local currents is considered to lead to a structural change in a local area of the membrane, and this change decays with a time constant that determines the refractory period.

Finally, it appears useful to contrast the physical mechanism suggested here with an explanation based on a chemical carrier mechanism that has been suggested to account for the sudden increase in Na^+ permeability of axons. Low temperatures might be expected to decrease the rate of reaction of Na^+ with the carrier and ultimately to make excitation impossible. It is difficult to see why narcotic molecules, when they interacted with the carrier should produce no obvious effect but that now there should be a sharp inactivation of carrier at a temperature 6 or more degrees higher than that taking place in untreated fibers. On the basis that the function of the blocking agent is to condense the membrane structure to an extent such that increased Na^+ permeability can no longer be brought about by the local circuit action responsible for propagating impulses, a sharp temperature dependence is to be expected only if the change in membrane structure is analogous to a phase transformation (liquid-solid). The uptake of certain narcotics by the membrane, and a reduction in temperature of nerve both lead to the same sort of molecular change within the membrane, e.g., a diminution of the free volume between the units of membrane structure, an enhanced interaction between such units, and hence a tendency toward a phase transition from liquid to solid. The alteration of the refractory period in the direction of a pronounced prolongation is also understandable from physical considerations if the enhanced interaction of elements of the membrane leads to a higher membrane viscosity, and hence to a prolonged time constant for recovery of the membrane after mechanical disturbance. That membrane viscosity changes may account for the effects of low temperature on nerve was suggested earlier by Gasser ('31). The nature of the physical disturbance in the membrane that may be occasioned by excitation has already been discussed in detail. (Mullins, '56).

SUMMARY

Concentrations of chemically inert blocking agents that do not diminish the amplitude of the action potential of frog sciatic nerve cause a marked prolongation of the refractory period of the fibers. A reduction of the ambient temperature does likewise and these two treatments interact. Treatment of the nerve with small amounts of blocking agents leads to a conduction block 6 or more degrees higher than the temperature at which conduction is normally blocked (2°C).

The sharpness of the action potential amplitude change with temperature suggests that the process responsible for the temperature sensitivity of conduction is analogous to a phase change in the membrane structure. The significance of this point is discussed.

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THE EFFLUX OF POTASSIUM FROM YEAST CELLS INTO A POTASSIUM FREE MEDIUM[1]

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SIX FIGURES

The movements of potassium into and out of the yeast cell have been extensively investigated (see reviews of Rothstein, '55, and Conway, '55). However, nearly all of the measurements represent the net movements of potassium based on chemical analysis, with individual contributions of influx and efflux to the net effect determined in only a few cases, by the use of isotope technique with K^{42}. The first isotope study (Hevesy and Nielsen, '41) revealed that in rapidly fermenting yeast a rapid exchange of K^+ takes place in addition to a rapid net uptake of K^+, whereas in slowly fermenting yeast, both processes are markedly diminished. In yeast without substrate, the K-exchange is exceedingly slow if O_2 is excluded, but proceeds at an appreciable rate if O_2 is present (Conway, '54; Rothstein, '56).

Recently, the K-efflux and K-influx in fermenting yeast have been determined by the cell column technique, which allows measurements of K-uptake and exchange at a constant potassium concentration (Rothstein and Bruce, '58). With K-free media, the K-efflux proceeds at a constant rate. With a high K-medium, the rate of influx is very much higher than the rate of efflux and is therefore the determining factor in net

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[1] This paper is based on work performed under contract with the U.S. Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

movements of K^+. With low or intermediate concentrations of K^+, the influx proceeds at a constant rate, but the rate of efflux increases after a lag period. In consequence, the K-uptake observed initially, ceases after 15 to 30 minutes, and in some cases, is followed by a period of K-loss.

With K^+ present in the medium, the rate of K-efflux can only be readily determined by isotope technique, attended by all of the difficulties associated with the short half-life of K^{42}. In K-free media the cells lose K^+ rapidly. In a minute or two a steady state is established in which the efflux is equal to the influx (Rothstein and Bruce, '58), with no opportunity to measure the initial rate of efflux. By the use of the cell column technique, however, the cells are continuously exposed to essentially K-free medium. Almost no influx can occur and the rate of efflux can be determined directly by chemical analysis of the effluent medium. In the present studies the efflux of K^+ against K-free medium has been determined by the above technique under a variety of conditions. The variables studied include, anaerobiosis, substrates, temperature, electrolytes, and inhibitors, including azide, cyanide, dinitrophenol, mercurials and redox dyes.

METHOD

The cell column technique has been described in detail in a previous paper (Rothstein and Bruce, '58). Briefly, the washed cells of baker's yeast (Standard Brands) were deposited in an even layer on the scintered glass disc of a 30 ml Buechner funnel (medium porosity, Pyrex, Corning no. 36060M), and covered with a filter paper and glass wool to prevent channeling in the yeast as fluid passed through. K-free medium under air or N_2 pressure was continuously passed through the cell column with the rate regulated by the pressure and by the amount of yeast on the column.[2] Samples of the effluent medium were collected continuously in a fraction collector and analyzed for K$^+$ in a flame photometer. The temperature was 25°C and the conditions aerobic, unless otherwise stated. In most experiments no buffer was used. The pH was adjusted with HCl to an initial value (3.5 unless otherwise stated). In a few experiments, triethylamine, succinate, tartrate (TST) buffer was used (Rothstein and Demis, '53).

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[2] In most experiments the amount of yeast on the column was 600 mg with an air or N_2 pressure of 5 to 7 pounds. The rate of flow of medium was 3 to 5 ml/min.

After passage through the yeast column, the medium contained K$^+$ in a concentration range of 1×10^{-6} to 1×10^{-5} M in most cases. The solution was evaporated to 1/10 of its initial volume before analysis so that the final concentration of K$^+$ was 1×10^{-5} or greater, within the accurate range of the flame photometer used for analysis (Wechselbaum-Varney type, manufactured by Fearless Camera).

Fresh baker's yeast (Standard Brands) was thoroughly washed and starved with aeration for several hours before use.

RESULTS

Yeast cells continuously exposed to K-free medium in air, lost K$^+$ at a relatively constant rate for long periods of time. At pH 3.5, the rate was about 10mM/kg of yeast/hr. In the presence of certain substrates, the rate was markedly increased (table 1). For example, with glucose the efflux was increased over 100%, but with fructose the increase in the rate was less than 50%. With pyruvate the rate of efflux was

TABLE 1

The effect of substrates on the efflux of K_+

The substrate concentrations were 0.1 M, unless otherwise stated

SUBSTRATE	RATE OF K-EFFLUX IN mM/kg/hr.	SUBSTRATE	RATE OF K-EFFLUX IN mg/kg/hr.
None	10.2	None	10.6
Glucose	21.5	.0001 M glucose	12.0
Fructose	14.4	.0005 M glucose	13.7
Pyruvate	16.8	.005 M glucose	23.2
Lactate	6.0	.05 M glucose	20.4
Ethyl alcohol	8.6	.10 M glucose	22.5

increased, but with lactate or ethyl alcohol the rate was decreased. Yet each of these substrates is respired by yeast at about the same rate. The maximal effect, in the case of glucose, was observed with a concentration of 0.005 M which induces a rate of respiration about ½ of maximal (Hurwitz and Rothstein, '51).

One comparison of aerobic and anaerobic conditions has already been made (Rothstein, '56). In the absence of substrate, the K-efflux under anaerobic conditions was reduced to a very low level (less than 1 mM/kg/hr. compared to 10 mM/kg/hr. in air), but in the presence of glucose, the rate was the same under both aerobic and anaerobic conditions (about 20 mM/kg/hr).

TABLE 2

The effect of temperature on the efflux of K^+

The pH was 4.0, the glucose concentration 0.1 M

TEMPERATURE °C	RATE OF EFFLUX IN mM/kg/hr.	
	No substrate	Glucose
5	1.7	2.5
15	5.1	7.2
25	9.7	14.5
35	10.6	17.5

At low temperature the rate of K-efflux was markedly decreased (table 2) but the temperature coefficient varied considerably with the actual temperature. Between 5° and 15°C, there was a three-fold increase in efflux, whereas between 25° and 35° there was only a slight increase. In a few experiments with glucose present, there was actually a decrease in the rate of efflux with the temperature raised from 25° to 35°C.

The pH of the medium was an important factor influencing the rate of K-efflux (fig. 1). The efflux was considerably higher when the pH of the medium was below 4.5, either with or without glucose, although no direct proportionality with H^+ concentration was evident. In the experiments without glucose, the medium always became more alkaline with shifts in pH of 0.2 to 0.3 unit in the range 2.0 to 4.0, and as much as 1.5 at pH 7.0. Back-titration to the initial pH indicated that for each mol of K^+ leaving the cell, about a mol of H^+ was absorbed. In the presence of glucose, the K^+-H^+ relationship was obscured by the excretion of organic acids, products of metabolism.

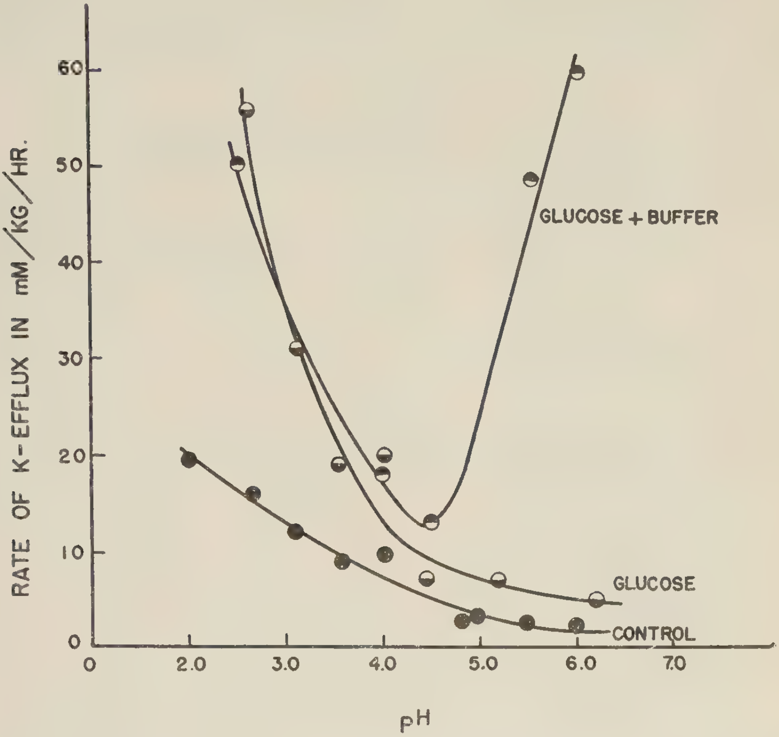

Fig. 1 The effect of pH on K-efflux with and without glucose and buffer; 500 to 800 mg of yeast, glucose 0.1 M, TST buffer 0.02 M.

The shifts in the pH of the effluent medium could be largely eliminated by the use of triethylamine-tartrate-succinate (TST) buffer, which is metabolically inert in the acid range (Rothstein and Demis, '53). Although the buffer did not influence the efflux to an appreciable extent in the pH range 2.0 to 4.5, it caused a tremendous increase at pH values above 4.5 (fig. 1). The effect was associated with the presence of the buffer cation rather than the anions, for triethylamine chloride or NaCl had as great an effect as the TST buffer.

The influence of cations particular Na^+, on K-efflux was investigated at different values of pH and of cation concentration. In each case the anion used was chloride, to which the yeast cell is relatively impermeable. Two kinds of response were observed, the first, an increased rate of K-efflux at higher values of pH and at higher concentrations of NaCl and the second, a decreased efflux at lower values of pH and at lower concentrations of NaCl. For example, at pH 4.5, with glucose present, the rate of efflux was increased, but at pH 3.5 and 2.5 it was decreased (table 3). At pH 3.5, concentrations of NaCl as low as .001 M, exerted a depressing effect (fig. 2). At pH 5.0 to 7.0, a marked increase in the K-efflux was observed, of the same magnitude as that with TST buffer (fig. 1).

TABLE 3

Effect of NaCl on K-efflux at different values of pH

The concentration of glucose was 0.1 M and NaCl, 0.05 M

pH	NO GLUCOSE		GLUCOSE	
	Control	NaCl	Control	NaCl
2.5	13.5	8.4	40.0	34.0
3.5	8.3	4.0	18.0	19.5
4.5	3.5	3.0	6.5	19.0

TABLE 4

The effects of different cations on K-efflux

The salt concentrations were 0.02 M and glucose 0.1 M

SALT	NO GLUCOSE	GLUCOSE
None	10	25
Choline Cl	10	26
LiCl	8	27
NaCl	2	10
$CaCl_2$	9	12
$CaCl_2$; NaCl	1	2
$MgCl_2$	10	18
$MnCl_2$	9	20

The depressing effect of NaCl at pH 3.5 is a fairly specific one. None of the other univalent ions tested (table 4) pro-

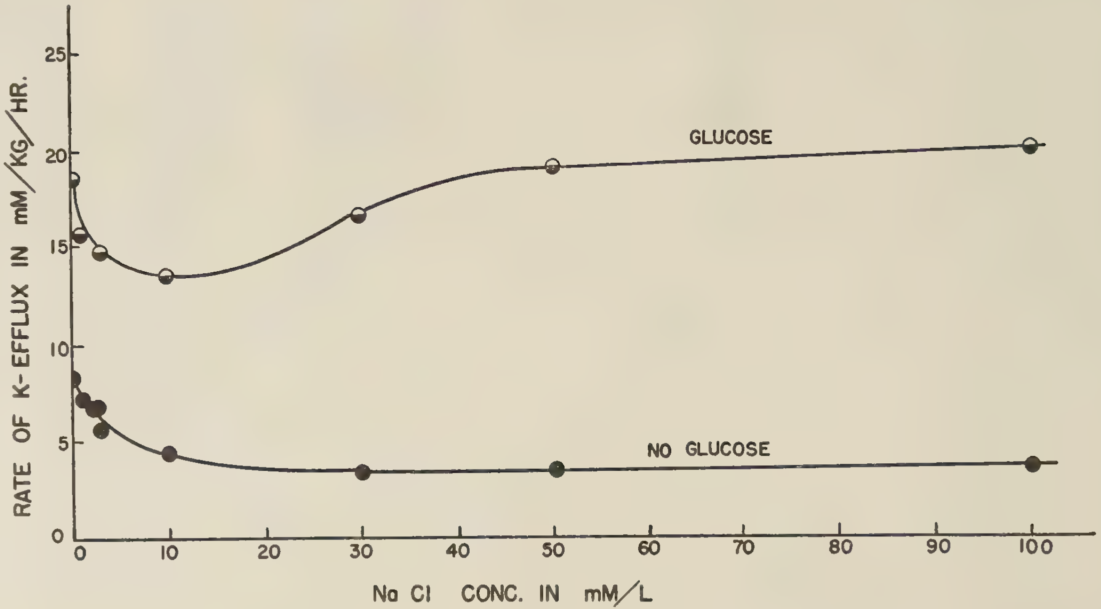

Fig. 2 The inhibition of K-efflux by Na^+; 600 mg. of yeast, pH 3.5, glucose 0.1 M.

duced the effect in the absence of glucose. However, calcium prevented the increased efflux associated with glucose. If both sodium and calcium were present the rate of K-efflux was very low.

A number of metabolic inhibitors are known to influence the uptake of potassium. Of these, azide has been studied most extensively (Conway, '54; Foulkes, '56). A dramatic but transient increase in K-efflux was induced by 2×10^{-3} M NaN_3

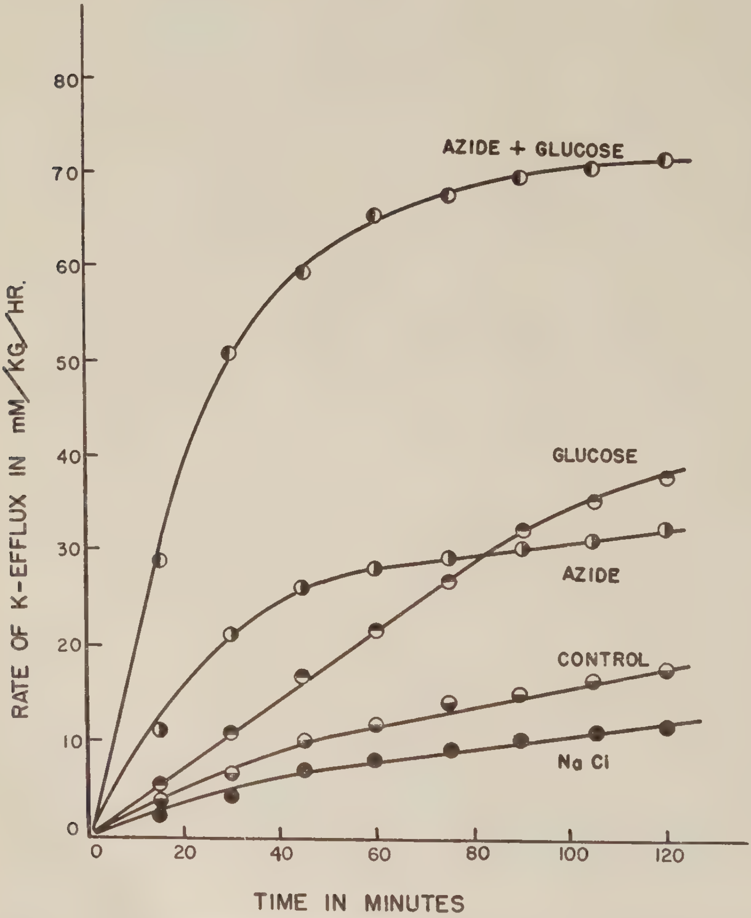

Fig. 3 The enhancement of K-efflux by azide; 600 mg of yeast, pH 3.5, sodium azide and sodium chloride 2×10^{-3} M, glucose 0.1 M.

(fig. 3), both in the presence and absence of glucose. In each case the rate of efflux returned to a low level after 45 to 60 minutes. In contrast sodium cyanide increased the rate of efflux only in the presence of glucose (fig. 4), and the effect was not as great as for azide. In the absence of glucose, there was a decreased efflux with NaCN of the same order as for NaCl at the same concentration. Dinitrophenol (DNP) at 1 × 10^{-3} M behaved like azide but at a lower concentration (1 × 10^{-4} M) the efflux was increased only in the absence of glucose.

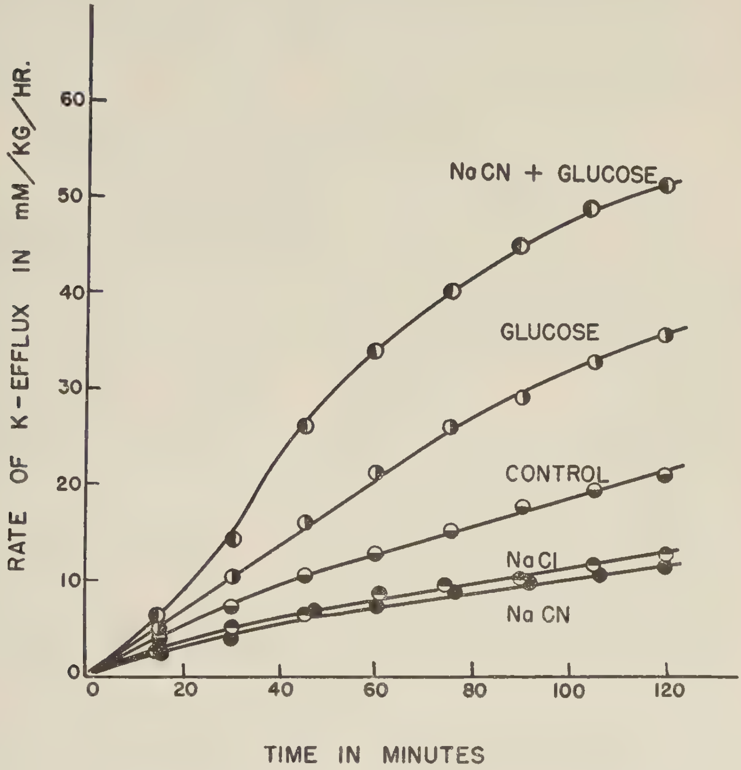

Fig. 4 The enhancement of K-efflux by cyanide; 600 mg of yeast, pH 3.5, sodium cyanide and sodium chloride 2 × 10^{-3} M, glucose 0.1 M.

Conway and Kernan ('55) has recently demonstrated that certain redox dyes could markedly influence the uptake of potassium although the rate of metabolism was not altered. A number of the same dyes plus others were tested for their effects on K-efflux. The dyes included a variety of chemical structures and a wide range of redox potentials (table 5). Each dye had some effect on the rates of fermentation and respiration, in most cases causing a small inhibition, but the magnitude of the effect being unrelated to the redox potential. In the case of the K-efflux, however, there was a definite correlation with redox potential. All of the dyes possessing a potential more negative than 0.05 to 0.10 except gallocyanine caused an immediate 500- to 1000-fold increase in the rate of efflux, which continued for some 30 minutes until 50 to 80% of the cellular potassium had leaked out. The rate then slowed somewhat. Gallocyanine had a borderline effect producing an increased K-efflux after about an hour. None of the dyes of potential more positive than 0.10 had any effect on K-efflux.

TABLE 5

The effects of redox dyes on K-efflux and on metabolism

The dye concentration was 1×10^{-4} M and glucose 0.05 M

DYE	REDOX POTENTIAL	FERMENTATION % OF CONTROL	RESPIRATION % OF CONTROL	K-EFFLUX mM/kg/hr.
Neutral red [1]	—.30 to —.35	50	48	> 100
Safranine [1]	—.25 to —.30	83	70	> 100
Nile blue [2]	—.10 to —.15	7	10	> 100
Methylene blue [1]	.110 to —.05	85	65	> 100
Thionine [1]	.05 to .10	80	70	> 100
Brilliant cresyl blue [1]	.045 to .10	65	27	> 100
Gallocyanine [1]	.021 to .10	95	80	12 [3]
Benzenoneindo 3′-chlorophenol [2]	.20 to .25	105	109	8
2,6-dichlorophenol indophenol [1]	.20 to .25	51	60	11
2,6-dibromoqinone-p-chloroimide [2]	> 30	—	—	10

[1] National Aniline Division, Allied Chem. and Dye Corporation.

[2] Matheson Coleman Bell.

[3] After one hour the rate of efflux increased to about 25 mg/kg/hr.

Additional studies with methylene blue indicated that the concentration of dye required to increase the K-efflux was between 1 × 10^{-5} M and 3 × 10^{-5} M (fig. 5). The effect was observed in the presence or absence of glucose or in the presence or absence of O_2. If the dye was left in contact with the cells for 30 minutes and then replaced by dye-free medium, the enhanced rate of K-efflux was not diminished.

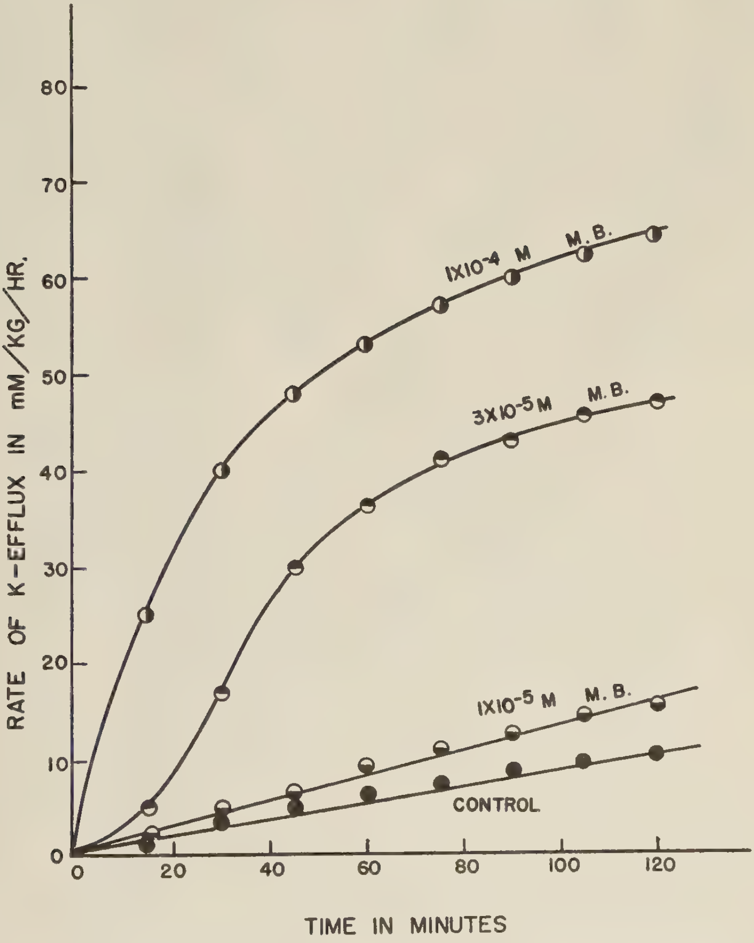

Fig. 5 The enhancement of K-efflux by methylene blue; 600 mg of yeast, pH 3.5, no glucose.

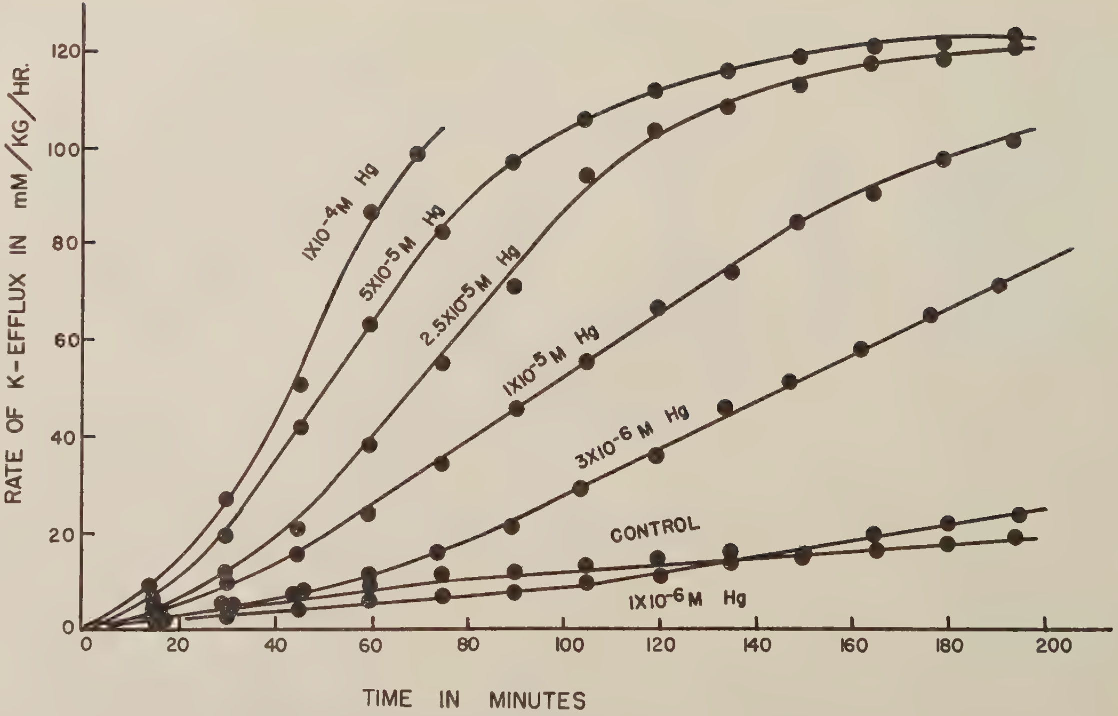

Fig. 6 The enhancement of K-efflux by mercuric chloride; 600 mg of yeast, pH 3.5, no glucose.

Another agent which dramatically increased the K-efflux was mercuric chloride (fig. 6). Concentrations as low as 1×10^{-6} M produced a small effect after a lag period of about 2 hours. With increasingly higher concentrations of Hg, the lag period was diminished and the rate of efflux was increased. With the highest concentration of mercury (1×10^{-4} M) the rate was so rapid, that 80% of the cellular K was lost in one hour. The effect of Hg was as great or greater in the absence of glucose as in its presence and the effect once established could not be reversed by cysteine, although cysteine if present initially, could prevent it.

An organic mercurial, p-chloromercuribenzoate also increased the K-efflux. This agent is poorly soluble at pH 3.5. However, at pH 4.5 it could be dissolved to the extent of 3×10^{-5} M, and it produced a 300% increase in the efflux. The same concentration of $HgCl_2$ produced a 600% increase.

Uranyl ion which acts on the cell surface to block sugar uptake (Rothstein, '54) had no appreciable effect on K-efflux.

DISCUSSION

The efflux of K^+ in exchange for H^+, involves the movement of K^+ from a K-rich cell (0.12 to 0.16 M/kg of cells) to an essentially K-free medium, and the movement of H^+ from an H-rich medium (pH 3.5 to 4.0) to an H-poor cytoplasm (pH 6.0 approx.). (Conway and Downey, '50). The large difference in the activities of K^+ and H^+ in the two compartments probably provides the driving force for the ion movements. If such is the case, the differences in the rates of efflux observed in the present experiments can be attributed to one of two causes, (1) a change in the driving force or (2) a change in the resistance of a membrane barrier to cation penetration. Certain of the factors studied, seem to definitely belong in the first of these categories. For example, the increased K-efflux at low pH can be attributed to an increase in the activity gradient of H^+ across the cell membrane. Similarly, the diminished rate of K-efflux after the first hour in the experiments with high concentrations of mercury can be attributed to a diminished K^+-gradient due to the large losses of cellular K^+. One other observation belongs in this category. When the pH of the medium is above 4.5, Na^+ or $(C_2H_5)_3N^+$ in high concentrations can markedly enhance the K-efflux. With the H^+ concentration very low, the other cations can substitute in the exchange reaction for K^+, and the gradient of Na^+ or $(C_2H_5)_3N^+$ becomes a factor. (The entrance of Na into the cell has been demonstrated by analysis). At pH 4.0 and below, however, the cells are relatively impermeable to Na^+ and $(C_2H_5)_3N^+$, and H^+ is the primary exchangeable ion.

All of the other observed effects on K-efflux can be attributed to changes in the resistance of a cation diffusion barrier, some of whose properties can be inferred from the nature and variety of substances which act upon it. In the first place, the magnitude of resistance of the barrier is not a fixed property, but can vary over an extremely wide range. The rate of efflux may be as low as 0.2 to 0.3 mM/kg/hr. at low temperature with no O_2; as high as 60 mM/kg/hr. with glucose at pH 2.5; and as high as 150 mM/kg/hr. in the presence of methylene blue, even though the dye doesn't interfere seriously with the metabolism of the cell, or in fact kill the cell (in terms of its failure to recover and reproduce). The regulation of the cellular potassium content depends on both the rate of influx, and of efflux. It has already been demonstrated (Rothstein and Bruce, '57) that variation in the rate of efflux is an important factor in determining the rate of K-uptake, especially at low concentrations of potassium. The action of x-ray on K-uptake by yeast, is in large part associated with an increased rate of K-efflux (Bruce and Stannard, '57). The present studies suggest that the mechanism by which inhibitors such as azide, DNP, mercury and redox dyes can block a net uptake of potassium may in large part be associated with their effects on the rate of efflux.

Certain properties of the cation barrier can be inferred from the nature of the substances and conditions which alter its properties. The wide variety of substances which are active suggests that they do not all act via a single mechanism. Furthermore some of the actions seem to be definitely linked to the metabolic state of the cell, whereas others are not. Although no complete explanation of all of the observed effects can be offered, certain inferences can be made. For example, the action of the redox dyes depends not on their chemical structure, or on the presence or absence of exogenous substrate, but on the redox potential. Apparently a constituent of the cation barrier is itself capable of undergoing an oxidation-reduction reaction. In view of the fact that mercuric chloride and p-mercuribenzoate have a similar effect to that of the redox dyes, the action of both kinds of agents is probably associated with their effect on sulfhydryl groups, the mercurials acting by formation of a mercaptide (p-chloromercuribenzoate is a specific sulfhydryl reagent (Boyer, '54), and the redox dyes by disulfide- sulfhydryl transformation. The action of x-ray on K-efflux (Bruce and Stannard, '57), may be mediated by a similar mechanism. It is perhaps relevant that the cell wall of yeast contains proteins (Northcote and Horne, '52), one of which is especially rich in sulfhydryl groups that can be oxidized to disulfide linkages by ferricyanide and enzymatically reduced to sulfhydryl (Nickerson and Falcone, '56). A role for sulfhydryl groups in permeability has been occasionally suggested.

The mechanism of action of substrates, O_2, temperature, and the inhibitors, azide, cyanide and DNP is far from clear. Perhaps some or all of these agents act on the sulfhydryl groups in an indirect manner by altering the redox potential at surface of the cell. For example, yeast establishes a potential in the surrounding medium which is shifted markedly on the addition of glucose (Conway and Kernan, '55). Furthermore, the redox potential in a yeast suspension is markedly different under aerobic and anaerobic conditions as indicated by tests of methylene blue reduction. Any agents that alter the rate or mode of metabolism would presumably alter the redox potential of the suspension. However, cyanide and azide, by blocking the cytochrome system, reduce the cell to the anaerobic mode of metabolism (fermentation). Yet these agents markedly increase the efflux of K^+, whereas anaerobiosis decreases the efflux. Furthermore, azide and DNP increase the rate of efflux even though substrate is absent.

A more likely explanation can be based on the premise that the efflux of potassium involves the movement of the cation through channels normally associated with the active transport system (K-pump) used by the cell for the inward movement of potassium against its activity gradient. The K-pump is dependent on metabolic energy for its operation. Thus metabolic factors such as substrates, temperature, and O_2 might also influence the leakage. Inhibitors such as azide and dinitrophenol might act through their metabolic effects or by a direct action on the K-pump, as has been suggested (Conway, '56; Foulkes, '56). The specific inhibition of K-efflux by Na^+ cannot be readily explained on the basis of sulfhydryl groups, or metabolic effects (there are no changes observed). However, Na^+ is known to compete with K^+ for the K-pump (Conway, '54) and its action may be related to this effect.

SUMMARY

The cell column technique afforded an opportunity to determine the rate of K-efflux into a K-free medium under the influence of a variety of factors, including temperature, pH, substrate, electrolytes, O_2, metabolic inhibitors, redox dyes and mercurials. The mechanism by which some of the agents act is readily apparent, but in other cases no obvious explanation can be made. Because K-efflux is primarily a K^+-H^+ exchange, the rate of efflux is increased at low pH. At higher pH values, other monovalent cations such as Na^+ or $(C_2H_5)_3N^+$ can increase the efflux by exchanging for K^+. The exceedingly high rates of efflux in the presence of certain redox dyes or mercurials is probably associated with their interaction with sulfhydryl constituents of the cell membrane essential for K^+ retention. The pattern of behavior with respect to temperature, O_2, substrates and metabolic inhibitors although

complex, suggests that the passage of K^+ through the cell membrane involves channels that are under the influence of metabolic activities, perhaps associated with the sulfhydryl groups mentioned above. Another possibility is the efflux of potassium via channels closely associated or identical with the active transport system for potassium, a hypothesis which could account for the metabolic effects and also for the specific inhibitory effect of Na^+.

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COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

THE EFFECT OF URANYL NITRATE ON THE PERMEABILITY OF CHICKEN ERYTHROCYTES [1]

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TWO FIGURES

Rothstein and collaborators ('48, '51) showed with yeast cells that uranium salts combined with the cell surface and inhibited glucose metabolism by interfering with the glucose-transport system across the membrane. Barron et al. ('48) on the other hand postulated that uranium salts combined with protein in the cell membrane and so altered the rate of penetration of molecules into a cell. Hoffman ('52) reported briefly on the action of several uranium salts on the erythrocytes of dogfish, tautog and human. He observed clumping phenomena, shape changes and also changes in rate of penetration. The rate of exit of urea from dogfish erythrocytes was inhibited up to 200%, while its entrance was inhibited to a lesser extent. Uranium had no measurable effects on the rate of movement of water, thiourea, ethylene glycol or glycerol into or out of these cells. Using human and tautog red cells, the entrance of glycerol, but not that of ethylene glycol or water, could be slowed by uranium. Hoffman interpreted these data to indicate that uranium combined reversibly with protein in the cell membrane.

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[1] These experiments were performed in the Physiology Department, Florida State University, Tallahassee.

Incidental to a study of the Na and K content, some preliminary observations were made on the non-electrolyte permeability of chicken erythrocytes which had been incubated with uranium. These observations extend those of Hoffman mentioned above.

Chicken blood was drawn by cardiac puncture and heparinized. The whole blood was separated into two containers. Ringer-Locke was added to the control flask (2 ml/10 ml of blood) while a solution of uranyl nitrate in Ringer-Locke was added to the experimental flask (2 ml of 1.8 mg/100 ml uranyl nitrate to 10 ml of whole blood) . These cell suspensions were incubated at 37°C under aseptic conditions. After various periods of incubation, aliquots were removed and the permeability to glycerol and urea were tested using a photoelectric technique (Hunter, '53). Typical results are shown in figures 1 and 2.

The upper two curves in each figure show the rate at which the erythrocytes swell in a hypertonic solution of non-electrolyte in Ringer-Locke as the non-electrolyte penetrates following the initial rapid shrinkage which does not appear in the record. There is no marked difference between the rate of swelling in either glycerol or urea of the control cells (curve on left) as compared with those which have been in the presence of uranyl nitrate for 51½ hours. The lower curves in each figure show the rate at which the glycerol and urea leave the cells. The erythrocytes were allowed to come to equilibrium (10–15 minutes) in a solution of 0.6 M non-electrolyte in Ringer-Locke. An aliquot of these cell suspensions were then added to 1.5 × Ringer-Locke.

As the glycerol or urea diffused from the cells, water moved in the same direction and the cells shrank. A comparison of the experimental and control cells shows that this concentration of uranyl nitrate does not affect the rate of exit of either glycerol or urea from these cells.

These data plus those of Hoffman suggest that whatever influence uranyl ion may have on the surface of erythrocytes of various species, it is impossible to predict how the rate of entrance and exit of different non-electrolytes will be affected.

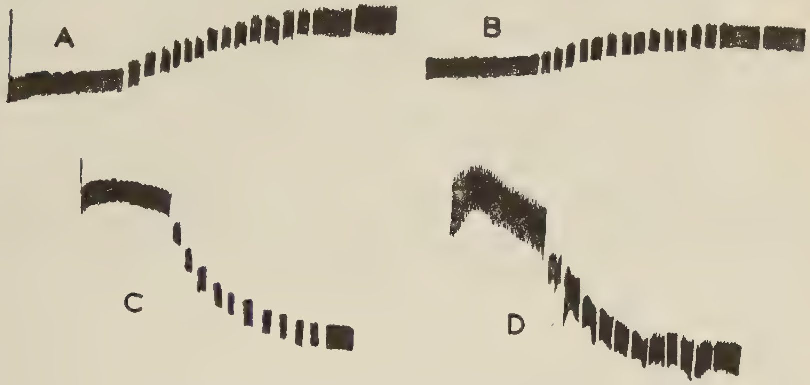

Fig. 1 Volume changes of chicken erythrocytes associated with the movement of glycerol. Continuous recording for first 15 seconds. Vertical breaks in curves every 15 seconds. Curves A and C — control; B and D — Cells incubated for 51½ hours in the presence of 7.2 × 10^{-6} uranyl nitrate. A and B — swelling in 0.3 M glycerol in Ringer-Locke; C and D — cells have been incubated in 0.6 M glycerol in Ringer-Locke, shrinking in 1.5 × Ringer-Locke.

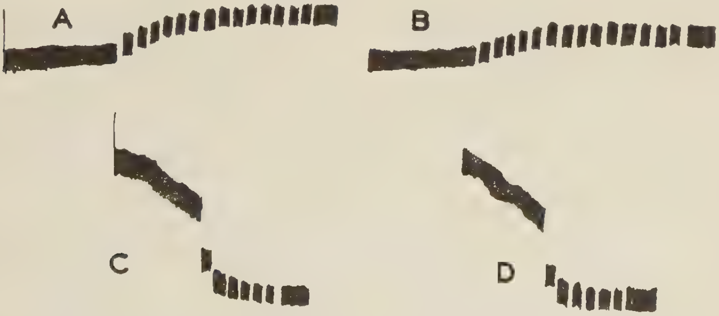

Fig. 2 Volume changes of chicken erythrocytes associated with the movement of urea. A—D — same as in figure 1 except urea substituted for glycerol.

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VOLUME CHANGES AND PARTIAL HEMOLYSIS IN HEAT-FRAGMENTED RED CELLS, AS OBSERVED BY INTERFERENCE MICROSCOPY

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ONE FIGURE

Partial hemolysis in heat-produced red cell fragments, as well as the decreases in volume of each resulting fragment which occur of necessity when a red cell breaks into several spherical fragments has already been described (Ponder, '49). The complexity of the situation, however, cannot be appreciated even by phase microscopy; volumes can be measured, but not the Hb concentration in individual fragments. Interference microscopy measures both.

METHODS

The results shown in figure 1 were obtained with a monocular AO-Baker interference microscope, a 100 × shearing-system water immersion objective with a corresponding condenser, a filar micrometer capable of being set to 0.1 μ, and monochromatic light ($\lambda = 5461$). A droplet of a suspension of washed human red cells, heated to 53°C for 3 minutes, was placed between a slide and a closely applied coverglass, vaselined along its edges. Measurements of the diameter of 30 spherical cells or fragments were made with the filar micrometer, and at the same time θ, the relative phase retardation produced by each fragment, was found with an accuracy of about ± 5 per cent. The concentration C of "apparent protein," principally terms of gm/100 gm is

$$C = \frac{0.84\ \theta}{t}$$

where t is the thickness (= diameter) of the cell or fragment.[1] Plotting C (ordinate) against the volume V of the cell or fragment (abscissa) gives figure 1, in which is also shown (circle within rectangle) the mean value of C and V for the unheated but spherical human red cell, together with their standard deviation (rectangle). Lines through the circle, parallel to the ordinate and to the abscissa, divide the diagram into 4 quadrants, experimental points appearing in three of these. Points above the line *ab* correspond to values of C greater than that found in the normal red cell, while points to the right of the line *cd* correspond to volumes greater than that of the normal red cell.

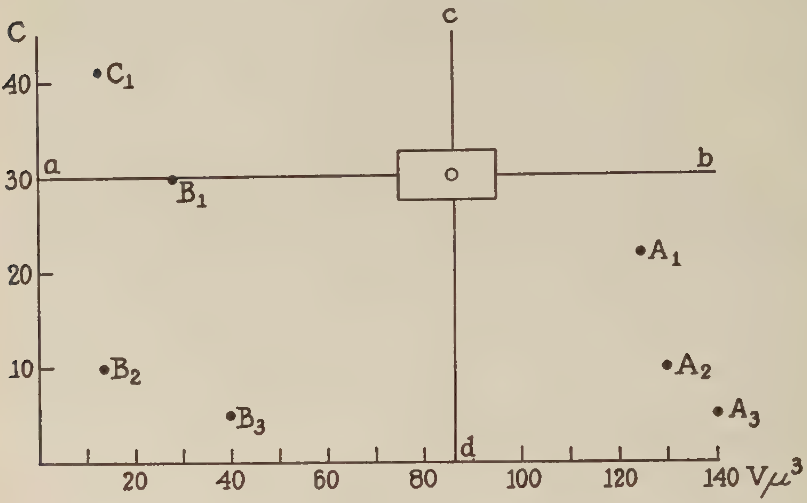

Figure 1

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[1] I have to thank Dr. Oscar W. Richards for verifying this expression and the calculations which depend upon it.

RESULTS

Consider the various possibilities.

I. Points in quadrant I would mean that the cell or fragment had undergone swelling and also that its concentration of Hb had increased. Since neither a cell nor a fragment can acquire Hb, there are no experimental points in quadrant I.

II. Points in quadrant II mean that the cell or fragment has undergone swelling but that the Hb concentration is reduced. The simplest case is that in which the reduction in C is wholly accounted by the increase in volume; in such a case $V_0/V_{cell} = C_{cell}/C_0$, where V_0 and C_0 are the volume and Hb concentration for the normal red cell, and where V_{cell} and C_{cell} are experimental values found for the volume and Hb concentration of the object in question. This relation is very nearly satisfied in the case of the point marked A_1. A more complex situation is one in which the cell or fragment swells, but in which there is partial hemolysis with a loss of a fraction p of the contained Hb. Here $V_0/V_{cell} > C_{cell}/C_0$, but as the concentration of Hb expected as the result of the swelling can be calculated easily, $C_{found}/C_{expected}$ can be ascertained, and shown to be equal to (1–p). For example, $p = 0.5$ in the case of the point marked A_2.

III. Points in quadrant III mean that cells have fragmented into small spherical fragments containing a lower Hb concentration than that present in the cell from which the fragments are derived. The diminished Hb concentration must be due to a loss of a fraction p of the initial Hb during the fragmentation process. The concentration of Hb found in the fragment can be compared with the concentration expected if no partial hemolysis had occurred; $C_{found}–C_0$ is equal to $(1–p)$, from which p can be calculated. For the point marked B_1, $p = 0.33$, *f*or the point marked B_2, $p = 0.67$, and for the point marked B_3, $p = 0.83$. The situation is either one in which there is no necessary shrinking or swelling, but only fragmentation without partial hemolysis (when $C_{frag} = 30$) or in which there are different degrees of partial hemolysis (when $C_{frag} < 30$).

IV. Points in quadrant IV mean that cells have fragmented into small spherical fragments containing a higher concentration of Hb than that in the cell from which the fragments are derived. This can be the result of a shrinking of the fragment (see Ponder and Barreto, '54, for a description of a similar shrinking phenomenon in red cell ghosts). There may be partial hemolysis as well. It there is not, V_{frag} can be measured and C_{frag}/C_0 can be calculated and called F; the shrinkage is then from FV_{frag} to V_{frag}. In the case of the point marked C_1, $F = 1.37$, and the fragment must have decreased in volume from 17.1 μ^3 to the observed 12.5 μ^3. If there is partial hemolysis at the same time, the situation is ambiguous, since a large value of p can be off-set by a large decrease in volume; the fraction $FV_{frag}/1-p)$, however, has a limit equal to V_0 for the cell from which the fragments are formed.

CONCLUSION

These results, obtained by interference microscopy, show that the phenomenon of red cell fragmentation by heat is more complex than has been thought to be hitherto. It has been realized that fragmentation may be accompanied by partial hemolysis, but that it may be accompanied by volume changes, other than those which concern the fragment and which necessarily occur when a red cell breaks into several fragments, is a new observation.

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THIS NUMBER COMPLETES VOLUME 51

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

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PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America